이학박사학위논문

중심체에서 히스톤 탈아세틸화 효소와 E3 유비퀴틴 중합효소의 기능 연구

Studies on the functions of histone deacetylases and E3 ubiquitin ligases in the centrosome

2018년 2월

서울대학교 대학원
생명과학부
박 선 아

중심체에서 히스톤 탈아세틸화 효소와 E3 유비퀴틴 중합효소의 기능 연구

Studies on the functions of histone deacetylases and E3 ubiquitin ligases in the centrosome

지도교수 이 건 수

이 논문을 이학박사 학위논문으로 제출함
2017년 12월

서울대학교 대학원
생명과학부
박 선 아

박선아의 이학박사 학위논문을 인준함
2017년 12월

위 원 장 _____(인)
부위원장 _____(인)
위 원 _____(인)
위 원 _____(인)
위 원 _____(인)

Studies on the functions of histone deacetylases and E3 ubiquitin ligases in the centrosome

A dissertation submitted in partial
fulfillment of the requirement
for the degree of

DOCTOR OF PHILOSOPHY

to the Faculty of

Department of Biological Sciences

at

Seoul National University

by

Seon-ah Park
December, 2017

Date Approved:

_____

_____

_____

_____

_____

Abstract

A centrosome consists of a pair of centrioles surrounded by pericentriolar materials (PCMs). During the cell cycle, the centrosome plays diverse roles in organizing cytoplasmic microtubules in interphase, assembling the mitotic spindle during mitosis and forming the cilium in quiescent cells. To carry out these functions, centrosomal proteins undergo diverse modifications such as phosphorylation, acetylation/deacetylation, and ubiquitination/deubiquitination. Here, I investigated the function of histone deacetylases in ciliogenesis and E3 ubiquitin (Ub) ligases in the regulation of centrosome duplication.

In chapter 1, I examined the function of class Ⅰ histone deacetylases (HDACs) in cilia assembly and elongation. HDACs are originally known to regulate gene transcription by deacetylating the histones. However, HDACs are also known to target and deacetylate non-histone proteins, thereby affecting their activities and functions. It has been known that HDAC1, 4, 10, 11 and SIRT1, 2 are localized to the centrosome and among them, HDAC1, HDAC5 and SIRT1 are involved in the suppression of centrosome amplification and HDAC6 is implicated in cilia disassembly. However, no HDAC has been identified as a positive regulator of ciliogenesis yet. Thus, I examined the role of class Ⅰ HDACs in the cilium assembly and elongation. The results revealed that HDAC3 and 8 are required for the assembly and elongation of the cilium.

In chapter 2, I investigated the function of E3 Ub ligases located at the centrosome among 226 E3 ubiquitin ligases. Selected centrosomal proteins should be

synthesized and degraded during the cell cycle. E3 Ub ligases are responsible for drastic degradation of centrosomal proteins during centrosome duplication Here, I identified E3 Ub ligases located in the centrosome. Among 226 E3 ligases, I found that 31 E3 ligases are located in the centrosome. Furthermore, I observed that FBXO31, a component of the SCF ubiquitination complex, is involved in the suppression of centrosome amplification in U2OS cells. On the other hand, ANAPC11, FBXL14, FBXO4, HERC3, KLHL2, PELI1, RNF135, SPSB4, and TCEB2 were implicated in the promotion of centrosome amplification.

Keywords: Centrosome, primary cilium, centrosome duplication, posttranslational modification, histone deacetylase, E3 ubiquitin ligase, cell cycle

Student Number: 2012-30885

Contents

Abstract ……...……..... **i**

Contents ……..... **iii**

List of figures ……...…….. **v**

Background and Purpose ……...…….. **1**

Background ……...……..... **2**

1. Centrosome ……..…… **2**

1.1.Structure of centrosome ……..... **2**

1.2.Function of centrosome ……...…..……. **2**

1.3.Centrosomal cycle ……...…..……. **3**

2. Cilia ……..... **3**

2.1.Structure of cilia ……..... **3**

2.2.Function of cilia ……..... **4**

2.3.Ciliogenesis ……..... **4**

2.4.Regulation of ciliary length ……..... ..... **5**

3. Histone deacetylases …….. **6**

3.1.HDAC …… **6**

3.2.HDACs in the centrosome …… **7**

4. E3 ubiquitin ligase …… **7**

4.1.E3 ubiquitin ligase …… **7**

4.2.E3 ubiquitin ligase in the centrosome…… **9**

Purpose …….. **16**

Chapter 1. Novel functions of histone deacetylases in the assembly and elongation of the primary cilium in serum-deprived cells

Abstract ……...…….……..... **19**

Introduction ……..……...……...… **20**

Materials and Methods ……..……...……... **23**

Results ……..……..... **27**

Discussion ……..……..... **53**

Chapter 2. Identification and functional analysis of E3 ubiquitin ligases in the centrosome

Abstract ……..……..... **57**

Introduction .....……...… …….. **58**

Materials and Methods ……..……..... **61**

Results ……..……..…..... **64**

Discussion ……..…… **74**

Conclusion ……..……..... **78**

References ……..……..... **79**

Abstract in Korean ……...……. **90**

List of Figures

Figure 1. The structure and function of the centrosome …… 10

Figure 2. Centrosome cycle ...……11

Figure 3. The primary cilium ……..12

Figure 4. Ciliogenesis ……..13

Figure 5. Histone deacetylases (HDACs) ……...……...14

Figure 6. Ubiquitination ……...……...15

Figure 7. Effects of TSA on the cilium formation ……...…33

Figure 8. Depletion of endogenous class Ⅰ HDACs and HDAC6 in RPE1 cells ...…….34

Figure 9. Involvement of the selected HDACs in primary cilia formation ……35

Figure 10. Depletion of endogenous class Ⅰ HDACs and HDAC6 in HK2 cells ...……...……...……...……...……...……...……...……...……..36

Figure 11. Involvement of the selected HDACs in primary cilia formation ……37

Figure 12. Flow cytometric DNA content analysis of HDAC-depleted RPE1 cells ……. ……. ……. ……. ……39

Figure 13. Involvement of HDAC8 in the cilium formation and elongation is not due to the effect of cell cycle arrest ……. ……41

Figure 14. Depletion of endogenous HDAC8 in RPE1 cells ……..42

Figure 15. HDAC8 is required for primary cilia formation …...……43

Figure 16. Depletion of endogenous HDAC8 and ectopic expression of HDAC8 in RPE1 cells ……. ……. ……. ……. …...45

Figure 17. HDAC8 deacetylase activity is not required for the cilium formation and elongation ...…….....……...…….....……46

Figure 18. HDAC8 is not required for centrosome splitting .....……..47

Figure 19. HDAC8 is not involved in cilium disassembly …….....….49

Figure 20. Localization of class 1 HDACs in ciliated RPE1 cells …...….....……. 51

Figure 21. Model of HDAC functions in ciliogenesis ...….....……...….....……52

Figure 22. Identification of known E3 ubiquitin ligases located in the centrosome ...……...……... 66

Figure 23. Expression of E3 ubiquitin ligases ……67

Figure 24. Identification of E3 ubiquitin ligases located in the centrosome ...……68

Table 1. List of centrosomal localization of E3 ubiquitin ligases ……. 70

Figure 25. Functional analysis of centrosomal E3 ubiquitin ligases for the suppression of centrosome amplification ……. 72

Figure 26. Model of E3 Ub ligase in the regulation of centriole duplication. …… 73

Background and Purpose

Background

1. Centrosome

1.1. Structure of centrosome

A centrosome consists of two centrioles that are surrounded by the pericentriolar materials (PCM). Two centrioles are perpendicular to each other. A centriole has 9 microtubule (MT) triplets and the mother and daughter centriole is ~450 nm and < 300 nm, respectively (Fig. 1A) (Bettencourt-Dias and Glover, 2007).

These make structural variations. Mother centriole differs from the daughter centriole in having distal appendage and subdistal appendage, which provides a docking site to plasma membrane for cilia formation and nucleates MT, respectively (Fig. 1A).

1.2. Functions of centrosome

The major function of the centrosome organizes MT arrays serving as microtubule organizing center (MTOC) in animal cells. It enables the centrosomes to regulate cell shape, migration, adhesion and polarity and cytoskeleton support in interphase. It serves as mitotic spindle pole to organize mitotic spindle, during mitosis. PCM proteins are recruited to the spindle pole and maturated to anchor and nucleate cytoplasmic MTs (Fig. 1B).

When cells become quiescent, the mother centriole differentiates a basal body to form the cilium. The basal body moves to plasma membrane and then its

distal appendages provide a docking site to nucleate and elongate the axoneme which is a microtubule-based core structure. The cilium transduces specific signals to cell body to regulate cellular metabolism such as cell growth and differentiation (Fig. 1B) (Fukasawa, 2007; Moser et al., 2009).

1.3. Centrosome cycle

A cell has one or two centrosomes. The number of the centrosome in a cell is thoroughly controlled. During S phase, a new centriole called a procentriole is formed adjacent to the mother centriole and it elongates by G2 phase. This calls centrosome duplication and centriole elongation. At the end of G2 phase, two centrioles move apart from each other toward cell body. This calls centrosome separation. After centrosome separation, PCM proteins are recruited to the centrosome and PCM volume expands 3~5 times compared to interphase. This calls centrosome maturation. This acquires the centrosomes to segregate chromosomes and divide cells during mitosis. During mitotic exit, mother-daughter centrioles are disengaged and separated. This step allows the centrosome to duplicate a new round of centriole in the next cell cycle (Fig. 2) (Nigg and Raff, 2009).

2. Cilia

2.1. Structure of cilia

Cilia are MT-based organelles that protrude from the cell surface and are conserved in eukaryotes. They are classified into three groups, motile, primary, and

nodal cilia. Motile cilia compose of nine pairs of outer MTs surrounding a single pair of inner MTs (9 + 2 arrangement). Primary cilia and nodal cilia both have an axoneme with a 9 + 0 MT arrangement. Primary cilia are immotile and ~ 0.2 μm in diameter and ~5 μm in length (Fliegauf et al., 2007).

2.2. Function of cilia

The Primary cilium is an antenna-like sensory organelle that protrudes from the cell surface of many differentiated cells. It is important for cellular sensing and signaling transduction from other cells or extracellular environments to the nucleus to elicit cellular responses such as cell growth, development, and differentiation. At cilia, there are components essential for signal transduction such as the Sonic hedgehog (Shh), platelet-derived growth factor receptor-α (PDGFRα), and Wnt signal pathway, which activate signaling pathway after sensing extracellular signaling at cilia (Michaud and Yoder, 2006).

Mutations of the genes encoding proteins responsible for the structure and function of the cilia induce disorders called ciliopathies such as cystic kidney disease, obesity, mental retardation, and retinal degeneration (Bettencourt-Dias et al., 2011).

2.3. Ciliogenesis

After mitogen deprivation or cell stress, cells exited the cells cycle. This is a requirement for forming the cilium. Golgi-derived distal appendage vesicles are accumulated in the vicinity of distal appendages of mother centrioles. Then the vesicles grow by fusion with ciliary vesicles on the distal tip of the mother centriole.

After then, the mother centriole converts to a basal body, proceeds with the elongation of MTs named as axoneme underneath the cap, and eventually culminates with the fusion of the nascent cilium with the cytoplasmic membrane (Sanchez and Dynlacht, 2016).

The cilium disassembly is triggered by the mitogen stimulation or interaction with Ca^{2+} and calmodulin (CaM), and tubulin deacetylation proceeds for regression of the ciliary axoneme (Plotnikova et al., 2012).

2.4. Regulation of ciliary length

When cilia elongate, cilia transduces length signal regulating cargo loading to the cytoplasm. During growing the cilium, cells use intraflagellar transport (IFT) to transduce ciliary components from cytoplasm to ciliary tip. IFT machinery moves to ciliary tip along elongating axoneme by anterograde kinsin-2 and cytoplasmic dynein (Pan and Snell, 2014). Upon IFT complexes reach to the ciliary tip by binding to ciliary cargo, the cilia are at steady-state. Steady-state assembly is balanced by continuous removal of axonemal subunits from the ciliary tip. Continuous assembly and disassembly of cilia at the ciliary tip maintain ciliary length at steady-state. Steady-state assembly requires IFT to provide axonemal subunits. When IFT-B is inactivated, the cargo is released and the cilia immediately start resorption. Subsequently, IFT-A complexes are activated and carry cargo releasing IFT-B complexes from the cilia to basal body (Ishikawa and Marshall, 2011).

3. Histone deacetylase (HDAC)

3.1. HDAC

Histone acetylation occurs at ε-amino group of lysine residue in the histones, neutralizes its positive charge, and alleviates the chromatin structure. This impedes outbreak of high-level chromatin structure and accesses to the target genes. Thus, histone acetylation affects structure and activity of target chromatin. On the one hand, the major function of histone deacetylases is to mediate a variety of biological procedures by inhibiting gene expression. This event occurs by removing acetyl group of ε-amino group of lysine residue in the histones, compacting chromatin (Haberland et al., 2009). HDACs also deacetylate and regulate activity, function, or stability of its target proteins by deacetylating non-histone proteins. For example, acetylated α-tubulin by α TAT1 is deacetylated by HDAC6 or SIRT2 (Pugacheva et al., 2007; Zhou et al., 2014). Deacetylated α-tubulin improves cell motility, induces MT depolymerization, and promotes remodeling of immune synapse which regulates cell signaling transduction and organizes synaptic adhesion (Serrador et al., 2004). Acetylated Ku70 by CBP and PCAF demolishes Ku70-Bax binding. The event allows mitochondrial localization of Bax to begin to cell death. Conversely, deacetylation of Ku70 by SIRT2 leads to prevent cell death by separating Bax from mitochondria (Cohen et al., 2004).

3.2. HDACs in the centrosome

Activity and stability of regulatory proteins which participate in centrosomal events are controlled by posttranslational modifications. Protein acetylation/deacetylation are the posttranslational modifications which regulate centrosomal events. Actually, a group reveals that some centrosomal proteins are acetylated. They report that HDAC1, HDAC5, and SIRT1 suppress centrosome duplication and amplification via a unique mechanism (Ling et al., 2012).

In case of the cilium assembly, α-TAT1 (MEC-17) acetylates cytoplasmic and ciliary MT at lysine 40. The acetylated MT is stabilized, and cilia are finally formed (Shida et al., 2010). During the cilium disassembly, aurora A phosphorylates and activates HDAC6 to destabilize MTs in the ciliary axoneme. HDAC6 deacetylates cortactin that is an actin polymerizing factor, and this reaction results in cilia disassembly (Ran et al., 2015). HDAC2 suppresses the cilium assembly in dividing pancreatic ductal adenocarcinoma cells. HDAC2 regulates expression of aurora A via histone deacetylase activity. Depletion of HDAC2 represses aurora A expression, resulting in cilia are formed in the dividing cells (Kobayashi et al., 2017).

4. E3 ubiquitin ligase

4.1. E3 ubiquitin ligase

Protein ubiquitination is a dynamic PTM involved in all aspects of eukaryotic biology. Ubiquitin proteasome system (UPS) governs process of cellular protein degradation, and regulates various cellular events such as cell proliferation, cell cycle progression, translation, and apoptosis (Zheng et al., 2016).

Ubiquitin is attached to substrate through a three-enzyme cascade catalyzed by E1, E2, and E3 enzymes. E1, an ubiquitin-activating enzyme, activates ubiquitin. E2, an ubiquitin-conjugating enzyme, catalyzes attachment of ubiquitin to the substrate, thereby transfers a moiety of activated ubiquitin to E3, ubiquitin-protein ligase. E3 confers substrate specificity to E2 by binding to both substrate and E2 (Glickman and Ciechanover, 2002).

Ubiquitinated proteins are recognized by receptors containing ubiquitin-binding domains (UBDs) and deubiquitinases (DUBs) (Swatek and Komander, 2016). After the ubiquitin molecules are attached to the substrate proteins, the ubiquitinated proteins are degraded by 26S proteasome complexes (Zheng et al., 2016).

Until now, more than 600 E3 ubiquitin ligases have been found in the human cells (Li et al., 2008). According to protein sequence homology, E3 ubiquitin ligases can be divided to HECT (homologous to the E6-AP carboxyl terminus) type, RING (really interesting new gene)-finger type, and RBR (ring between ring fingers) domain-containing ubiquitin ligases (Bedford et al., 2011; Deshaies and Joazeiro, 2009; Petroski and Deshaies, 2005). RING-finger type is the well-studied E3 ubiquitin ligases, which contains anaphase-promoting complex (APC) and Skp1-Cullin-F-box protein (SCF) complex (Jin et al., 2004). SCF complex consists of CUL1, Rbx1, Skp1, and F-box proteins (Peters, 2006; Zheng et al., 2002). Depending on the different binding domain, F-box proteins are divided into three subfamilies, the FBXW subfamily, FBXL subfamily, and the FBXO subfamily (Frescas and Pagano, 2008; Welcker and Clurman, 2008).

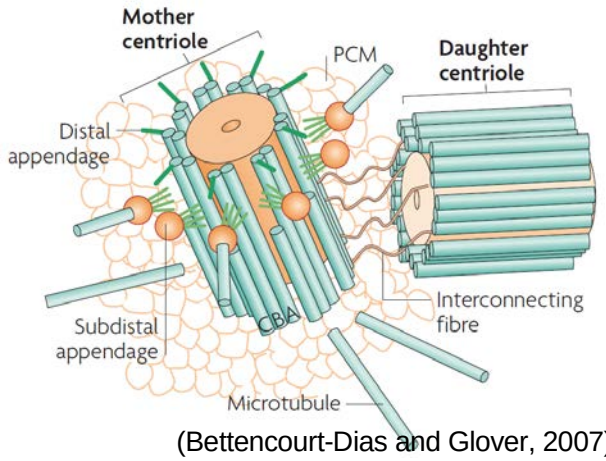
4.2. E3 ubiquitin ligase in the centrosome

During the centrosome cycle, centrosomal proteins undergo the process of drastic synthesis or degradation. The capacity of centrosomal E3 ubiquitin ligases to degrade centrosomal proteins in the cell cycle is important for proper progression through the centrosomal cycle, because they ubiquitinate and influence the activity of centrosomal proteins, thereby regulating centrosomal cycle.

PLK4 is an important regulator of centriole biogenesis. PLK4 auto-phosphorylates, which is important for regulating PLK4 stability and the centriole number. This auto-phosphorylation regulates the centriole number by controlling the degradation mediated by FBXW11/β-TrCP. Activity of Cullin1 (CUL1) is essential for the degradation of PLK4 (Guderian et al., 2010). The degradation of PLK4 by CUL1 induces the overexpression of cyclin E / CDK2, and regulates the stability of basal PLK4. There are biological meanings in that that limit the excessive centriole formation and reveal the mechanisms of cell division error mediated by the centrosomes (Korzeniewski et al., 2009). Mind bomb 1 (Mib1) binds to and ubiquitinates PLK4. Ubiquitinated PLK4 is degraded and rarely detected in the centrosome. This modification regulates the capacity to interact with PLK4 and (an) other centrosomal protein(s) and results in the inhibition of centriole over-duplication (Cajanek et al., 2015).

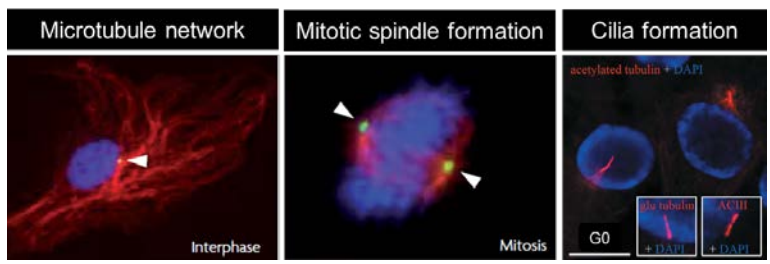
PCM1 promotes the formation of cilia by anchoring Mib1 to centriolar satellites and restricting it from centrioles. This leads to destabilize Talpid3, an interacting partner of Rab8a, via polyubiquitination, a failure to recruit Rab8a to centrioles, and inhibition of cilia formation (Wang et al., 2016).

A.



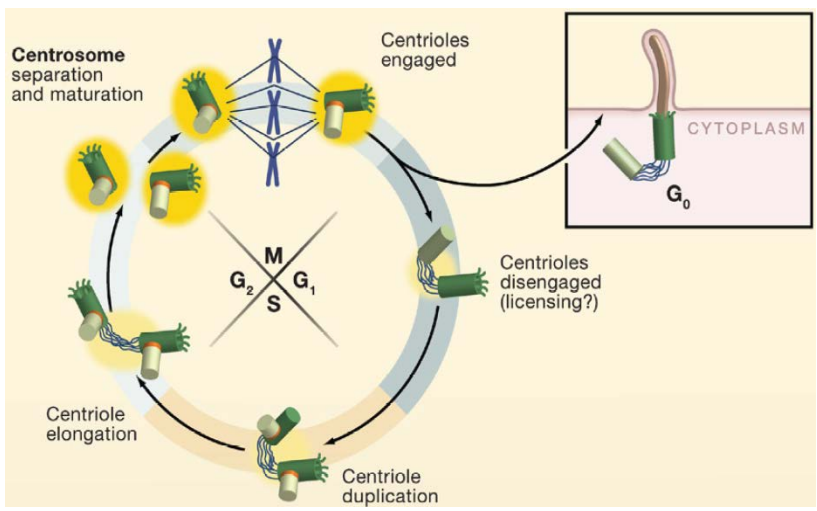
(Bettencourt-Dias and Glover, 2007)

B.



(Fukasawa et al., 2007, Moser et al., 2009)

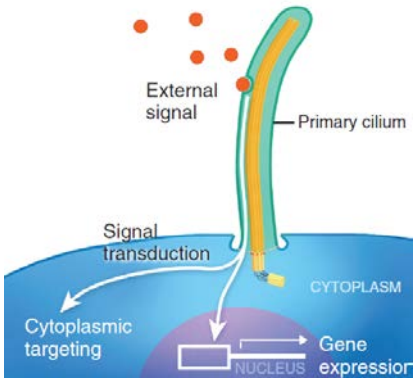
Figure 1. The structure and function of the centrosome. (A) A centrosome consists of two centrioles surrounded by PCM. Two centrioles, mother and daughter centriole, are perpendicular to each other. The mother centriole has distal and subdistal appendage, which provides a docking site for the cilium formation and functions in MT nucleation, respectively. (B) In interphase, the centrosome functions as MTOC which regulates cell shape, mobility, polarity, and cytoskeletal support. During M phase, it segregates duplicated chromosomes to the daughter cells by acting as mitotic spindle pole. In quiescent cells, the centrosome moves to plasma membrane and a mother centriole differentiates to a basal body to form the cilium.



(Nigg and Raff, 2009)

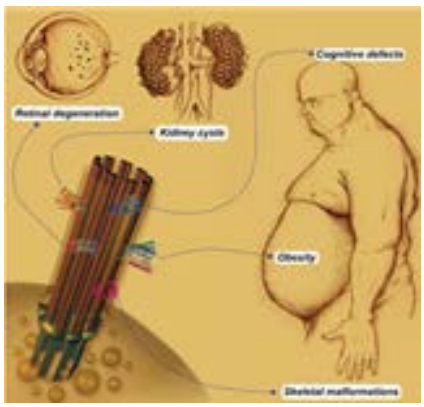
Figure 2. Centrosome cycle. Like chromosome, duplication and separation of the centrosome take place during the cell cycle. In late G1, each centriole starts to duplicate a procentriole perpendicularly. The procentriole is elongated along S and early G2 phase. In late G2, the centrosomes are separated and moves apart from each other and PCM proteins are recruited to the centrosome. The maturated centrosomes segregate duplicated chromosome to the daughter cells equally. After then, the centrosome is disengaged and prepares centrosome duplication in the next cell cycle.

A.



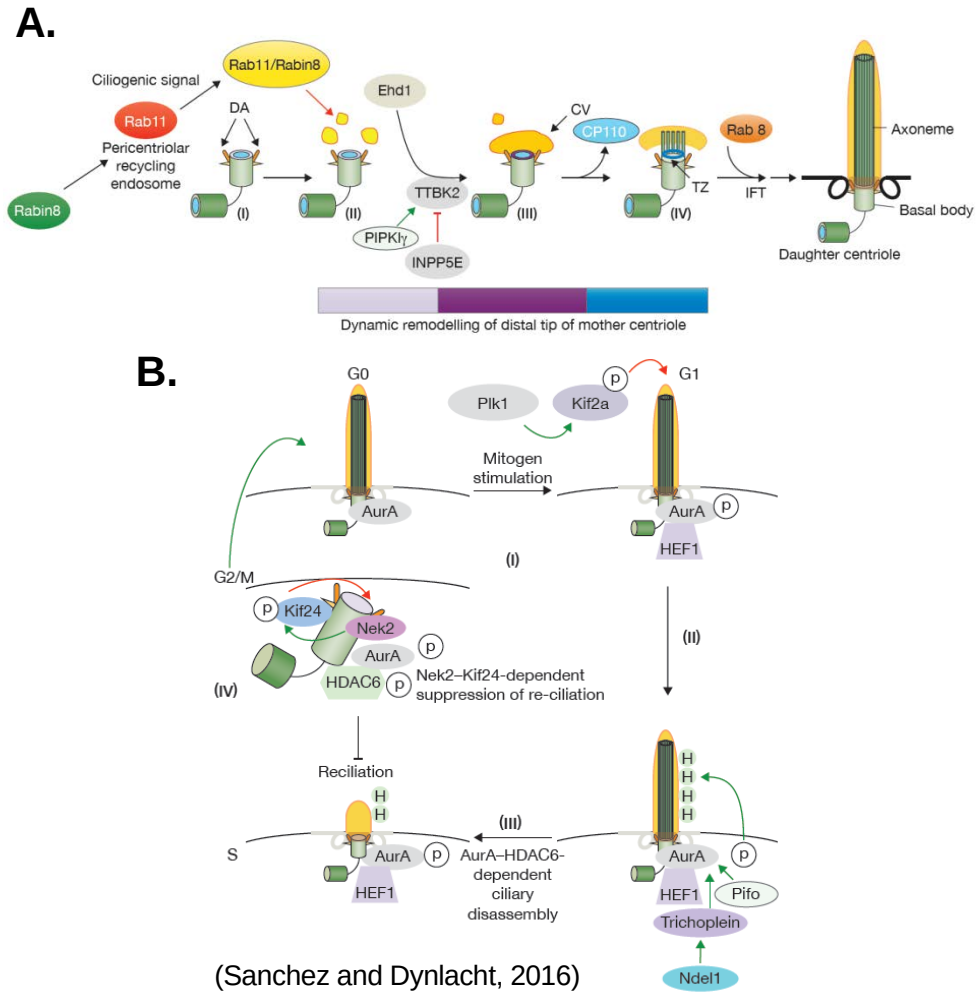
(Buchwalter et al., 2016)

B.



(Cell cover, Jun. 15, 2007)

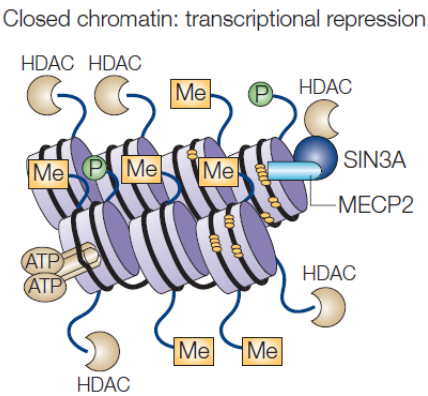
Figure 3. The primary cilium. (A) The cilium is a sensory organelle protruded from the cell surface. It receives some signal from extracellular environments and transduces the signal to the nucleus to evoke cellular events. It concentrates signaling pathway components. (B) Defects in structure and function of the cilium result in disorders called ciliophaties such as retinal degeneration, kidney cysts, cognitive defects, skeletal malformations, and obesity.



(Sanchez and Dynlacht, 2016)

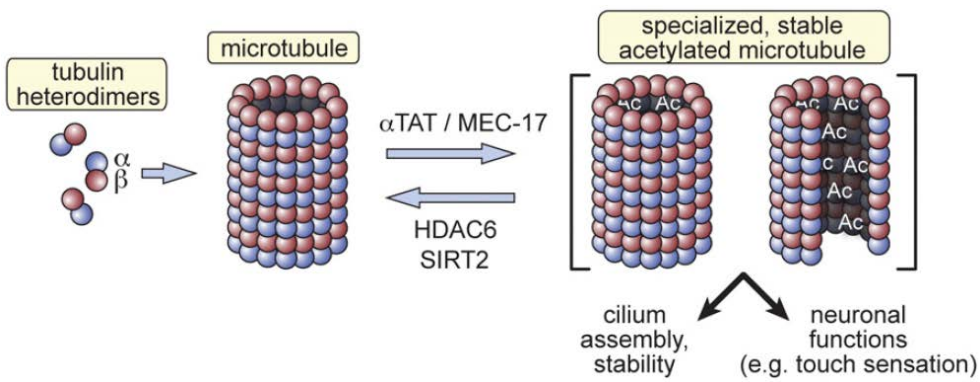
Figure 4. Ciliogenesis. (A) When cells exit the cell cycle, Golgi-derived distal appendage vesicles are accumulated in the vicinity of distal appendages of mother centriole. Then the vesicles are fused to ciliary vesicle, the mother centriole differentiates a basal body, and MT-based axoneme is extended underneath the cap. Finally, the basal body docks to the plasma membrane and is fused with cytoplasmic membrane. (B) Cilium disassembly is induced by mitogen stimulation. This event is governed by PTM mechanisms which control the activity and stability of ciliary proteins.

A.



(Johnstone et. al., 2002, *Nature Reviews Drug Discovery*)

B.



(Leroux, 2010)

Figure 5. Histone deacetylases (HDACs). (A) HDACs regulate DNA expression by deacetylating histones wrapping DNA. (B) HDAC6 and SIRT2 deacetylate α-tubulin, thereby promoting the cilium disassembly.

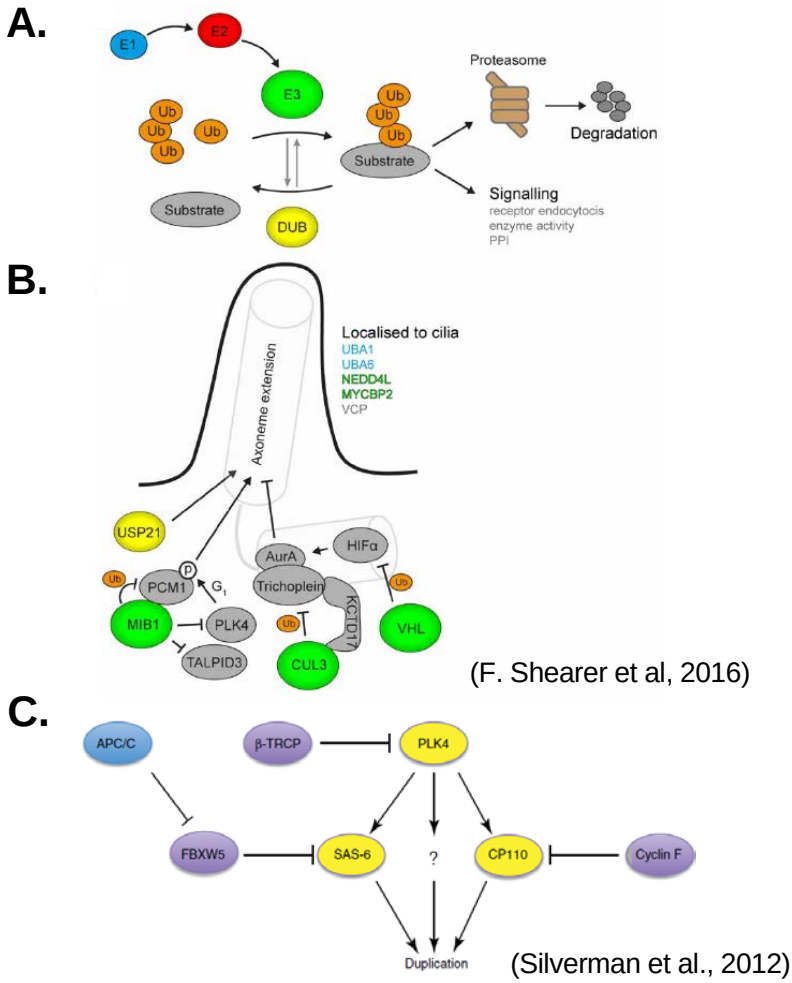


Figure 6. Ubiquitination. (A) Protein ubiquitination reaction is catalyzed by three enzyme cascade, E1, E2, and E3. E1 recognizes and activates ubiquitin in ATP-dependent manner, then transfers it to E2. E3 binds to ubiquitin-binding E2 and the substrate. E3-binding E2 catalyzes transfer of the ubiquitin to the substrate. These modifications evoke diverse cellular events, such as endocytosis and proteasomal degradation. (B-C) The ubiquitination events also take place in the centrosome. The reaction governs the centrosome cycle such as centrosome duplication and ciliogenesis.

Purpose

The centrosome functions as MTOC and controls cell migration, shape, subcellular transport, and cytoskeleton support in interphase. During mitosis, it functions as a mitotic spindle pole, which robustly nucleates and extends the bipolar spindles to segregate duplicated chromosomes to two daughter cells. In a quiescent G0 / G1 phase, the centrosome moves to the plasma membrane and differentiates to a basal body to assembly a cilium. The cilium becomes a platform of major signal transduction, which transduces signals to nuclei to elicit cellular responses such as cell growth, development, and differentiation. Defects in structure, functions, and elongation progress of the cilium induce abnormal signal transduction.

Protein phosphorylation is an important PTM that controls ciliogenesis. Also, it has been recently reported that the effects of histone deacetylases on the cilium disassembly. However, no histone deacetylases has been identified as a positive regulator of ciliogenesis. Thus, I attempt to examine the histone deacetylases that regulate assembly and elongation of the cilium.

The activity, stability, and function of centrosomal proteins are controlled by not only protein phosphorylation and acetylation/deacetylation but also ubiquitination/ deubiquitination. So far, about 600 E3 ubiquitin ligases have been found and the functions and mechanisms of some E3 ubiquitin ligases in the centrosome cycle and ciliogenesis were revealed. However, centrosome-related E3 ubiquitin ligases were mostly found by protein-protein interactions using immunoprecipitation (IP) combined with mass spectrometry, thus making the discovery slow. Given that the

importance of centrosomal E3 ubiquitin ligases in the regulation of centrosomal proteins throughout the cell cycle, it requires the more integrative approaches to find the E3 ubiquitin ligases and understand their function in the centrosome and cilia. Thus, I attempt to identify centrosomal E3 ubiquitin ligase among 226 E3 ubiquitin ligase plasmids and examine the functions of identified E3 ubiquitin ligases.

Chapter 1.

Novel functions of histone deacetylases in the assembly and elongation of the primary cilium in serum-deprived cells

Abstract

Primary cilia are extended from mother centrioles in quiescent G0/G1 cells and retracted in dividing cells. Diverse posttranslational modifications play roles in the assembly and disassembly of the cilium. Here, I examined class I histone deacetylases (HDACs) as positive regulators of cilia assembly in serum-deprived RPE1 and HK2 cells. I observed that the rate of primary cilium assembly was significantly reduced in HDAC3- and 8-depleted cells. Ciliary length was also shortened in HDAC8-depleted cells. A knockdown-rescue experiment showed that the deacetylase-dead mutant and wild type HDAC8 rescued the cilia assembly rate and ciliary length, suggesting that deacetylase activity may not be critical for HDAC8 function in cilia assembly and ciliary length control. This is the first study to report that HDACs are involved in the assembly and elongation of the primary cilium.

Introduction

The primary cilium is a non-motile, sensory organelle that protrudes from the cell surface. It transduces specific intercellular signals to the cell body and regulates various cellular events for growth and differentiation. Defects in ciliary structure and function lead to pleotropic disorders called ciliopathies, such as cystic kidney disease, obesity, mental retardation, and retinal degeneration.(Goetz and Anderson, 2010) The loss of cilium may be an early signature event during oncogenic transformation.(Seeley et al., 2009)

The primary cilium originates from a mother centriole in a quiescent G0/G1 cell. The first visible sign of ciliogenesis may be the accumulation of Golgi-derived distal appendage vesicles in the vicinity of distal appendages of mother centrioles (Sanchez and Dynlacht, 2016). Vesicular fusion produces a membranous cap on the distal tip of the mother centriole. The mother centriole then differentiates into a basal body and extends microtubules underneath the cap. Finally, the nascent cilium docks at the cytoplasmic membrane by fusion with the ciliary sheath, establishing continuity of these compartments.(Sung and Leroux, 2013) Disassembly of the primary cilium is triggered by the replenishment of serum growth factors, and tubulin deacetylation precedes for regression of the ciliary axoneme.(Li and Yang, 2015)

Diverse regulatory mechanisms control the assembly and disassembly of the primary cilium. Rab GTPases are implicated in the membrane trafficking of ciliary vesicles at the initial step of ciliogenesis (Li and Hu, 2011). After being recruited by BBSome, Rab8 drives the docking and fusion of exocytic vesicles to the base of the

ciliary membrane (Nachury et al., 2007). CEP164 helps dock vesicles through interactions with Rab8 and Rabin8 at the distal end of the mother centriole (Schmidt et al., 2012). Furthermore, Arl3, Arl6, and Arl13b represent another group of small GTPases that are implicated in ciliopathies (Li and Hu, 2011).

Protein phosphorylation is an important regulatory mechanism, and a number of protein kinases have been determined to play essential roles at specific steps of cilia assembly and disassembly (Sanchez and Dynlacht, 2016). Tau-tubulin kinase 2 (TTBK2) promotes the removal of CP110, which caps the mother centriole and promotes the recruitment of intraflagellar transport (IFT) proteins to build the ciliary axoneme (Goetz et al., 2012). MAP/microtubule affinity-regulating kinase 4 (MARK4) is another positive regulator of the early steps in ciliogenesis for the removal of CP110 prior to axoneme extension (Kuhns et al., 2013). In contrast, aurora A is a critical kinase for cilium disassembly. Aurora A phosphorylates a number of ciliary proteins, including histone deacetylase 6 (HDAC6), to induce microtubule destabilization (Pugacheva et al., 2007). PLK1 induces the microtubule-depolymerizing activity of kinesin superfamily protein 2A (KIF2A) to disassemble the primary cilium in a growth signal-dependent manner (Miyamoto et al., 2015). NEK2 also stimulates KIF24, another microtubule-depolymerizing kinesin, to prevent the outgrowth of cilia in cells entering S phase (Kim et al., 2015b).

Protein acetylation is another posttranslational modification that regulates the functions of the centrosome and cilia (Li and Yang, 2015). α-Tubulin acetyltransferase 1 (TAT1) and HDAC6 are responsible for the acetylation and deacetylation of α-tubulin, which regulates microtubule stability (Akella et al., 2010;

Pugacheva et al., 2007; Shida et al., 2010). During the cilium disassembly process, aurora A activates HDAC6 to destabilize microtubules in the ciliary axoneme (Pugacheva et al., 2007). Another example is HDAC2, which suppresses primary cilium formation in dividing tumour cells (Kobayashi et al., 2017). The involvement of deacetylases in centrosome function was first reported by Ling et al (2012) (Ling et al., 2012). Of 18 known lysine deacetylases (e.g., HDAC1-11 and SIRT1-7), 10 suppress centrosome amplification, and their centrosome amplification-suppressing activity is strongly associated with their ability to localize to centrosomes (Ling et al., 2012). Among them, HDAC1, HDAC5 and SIRT1 exhibit the highest suppressing activities likely via a unique mechanism (Ling et al., 2012). Thus far, no deacetylase has been identified as a positive regulator of ciliogenesis.

In this work, I examined deacetylases that function as positive regulators of primary cilia formation in serum-deprived cells. My results revealed that selected HDACs such as HDAC3 and 8 are required for the assembly and elongation of the primary cilium.

Materials and Methods

Cell culture and drug treatment

hTERT-RPE1 and HK2 cells were cultured in DMEM/F-12 (Welgene, LM 002-04) supplemented with 10% foetal bovine serum (FBS) (Welgene, S101-01) and Plasmocin™ (Invivogen, anti-mpt). For primary cilium assembly, the amount of FBS in the medium was reduced to 0.1%. TSA (Sigma, T8552) was added to RPE1 cells for 12 h to inhibit HDAC activity.

Transfection of siRNAs and plasmids

ST Pharm was used to synthesize siRNAs specific to *HDAC1* (*siHDAC1*; 5' – GCU UCA AUC UAA CUA UCA ATT – 3'), *HDAC2* (*siHDAC2*; 5' – CCC AAU GAG UUG CCA UAU AAU TT – 3'), *HDAC3* (*siHDAC3 #1*; 5' – CAA CAA GAU CUG UGA UAU UTT - 3' and *siHDAC3 #2*; 5' – GCA CCC GCA UCG AGA AUC ATT - 3'), and *HDAC8* (*siHDAC8*; 5' – GCU GGG AGC UGA CAC AAU ATT – 3'). siRNAs specific to *HDAC1* (L-003493-00-0005), *HDAC2* (L-003495-02-0005), *HDAC8* (M-003500-02-0005), and *HDAC6* (J-003499-05-0005) were purchased from Dharmacon. The control siRNA was scrambled (*siCTL*; 5'- GCA AUC GAA GCU CGG CUA CTT - 3'). The siRNAs were transfected into RPE1 and HK2 cells with RNAiMAX (Invitrogen, 13778-075).

The siRNA recognition site of *HDAC8* was silently mutated using the 5'-GCT AGG TGC CGA TAC CAT T -3' primer. The deacetylase-dead HDAC8 mutant includes a histidine substitution to alanine at residue 142 and 143. The HDAC8

constructs were subcloned into the *pcDNA5FRT-neo* vector. Plasmids were transfected into RPE1 cells with Fugene HD (Promega, E2311).

Antibodies

The primary antibodies used were specific to HDAC1 (Abcam, ab31263), HDAC2 (Abcam, ab12169), HDAC3 (Abcam, ab7030), HDAC8 (Proteintech, 17548-1-AP), HDAC6 (Abgent, AP1106a), acetylated α-tubulin (Cell Signalling Technology; 5335S, Sigma; T6793), Centrin-2 (Millipore, 04-1624), Rootletin (Sigma, HPA021191), Flag (Sigma, F3165), α-tubulin (Sigma, T6199-200UL), γ-tubulin (Santacruz, sc-7396), or GAPDH (Ambion, AM4300). The secondary antibodies used were conjugated to fluorescent dyes (Alexa-488, Alexa-555, Alexa-594, and Alexa-647; Life Technologies) or hydrogen peroxidase (mouse, Sigma; rabbit, Millipore).

Immunoblot analysis

Specific proteins were detected as previously described (Seo et al., 2015). In brief, cells were lysed with a single-detergent lysis buffer [50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 150 mM NaCl, and 1% Triton X-100 or NP-40] supplemented with protease inhibitor cocktail (Sigma, P8340) for 30 min on ice and centrifuged at 12,000 rpm for 20 min at 4°C. The supernatants were mixed with 5× sodium dodecyl sulfate sample (SDS) sample buffer [313 mM Tris-HCl (pH 6.8), 50 mM DTT, 10% SDS, 50% glycerol, and 0.05% bromophenol blue] and boiled for 10 min. Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with blocking buffer (5% skim milk in 0.1% Tween 20 in

TBS) for 1 hour, incubated with the indicated primary antibody for 16 hours, washed four times with TBST (0.1% Tween 20 in TBS), incubated with the appropriate secondary antibody in blocking buffer for 40 min and washed four additional times with TBST. Hydrogen peroxidase signals were detected using ECL solution (ABfrontier, LF-QC0101).

Immunofluorescence microscopy and ciliary length measurements

Cells were subjected to immunostaining as previously described (Kim et al., 2015a). In brief, cells grown on 12-mm coverslips were fixed with cold methanol on ice for 10 min, and permeabilized with PBST (0.3% Triton X-100 in PBS) for 10 min. Cells were blocked with blocking buffer (3% bovine serum albumin and 0.3% Triton X-100 in PBS) for 20 min, incubated with the indicated primary antibody for 1 hour, washed three times with PBST, incubated with the indicated secondary antibody for 30 min, washed three additional times with PBST, counterstained with DAPI solution for 1 min, and mounted onto glass slides with ProLong Gold antifade reagent (Invitrogen, P36930). Cells were observed under a fluorescence microscope (Olympus IX51) with a 60×/1.25 oil Iris (UFlanFl) objective lens. Images were acquired on a CCD camera (Qicam fast 1394, Qimaging) and analysed using ImagePro 5.0 (Media Cybernetics, Inc.). Ciliary length was measured using the NeuronJ plugin to ImageJ software (National Institutes of Health).

Cell cycle analysis

Cells were washed in PBS, trypsinized with tripsin/EDTA, and harvested into 15 ml tube. After centrifugation of cells at 1,200 rpm for 3 mins, the supernatant was removed. The cells were resuspended with 500 μl of PBS and fixed with 5 ml of cold ethanol for 4 hours at 4℃. After centrifugation of cells at 1,200 rpm for 3 mins, the supernatant was removed and the cells were resuspended with 3% BSA, recentrifugated, the supernatant were removed. The cells were stained with PI for 30 min at 37℃. Finally the cells were analysed with BD FACSCanto flow cytometer.

Statistical analysis

All experiments were repeated at least three times. GraphPad Prism 5 was used for the unpaired two-tailed *t*-test, and SigmaPlot 12.0 (Systat Software) was used for two-way ANOVA.

Results

Inhibition of cilia formation by trichostatin A

To investigate the involvement of HDACs in cilia formation, we examined the effects of trichostatin A (TSA), an inhibitor of class I HDACs, on cilia formation in RPE1 cells. Cells were treated with TSA for 12 h and cultured in serum-deprived medium for cilia formation (Fig. 7A). Cilia were visualized by immunostaining with the acetylated α-tubulin antibody (Fig. 7A). The rate of cilia formation was reduced following treatment with TSA in a dose-dependent manner (Fig. 7B). Furthermore, the ciliary length was also shortened (Fig. 7C). These results imply the involvement of class I HDACs in cilium assembly and elongation.

Inhibition of cilia formation with depletion of class I HDACs

I determined the cilia assembly rate and ciliary length in HDAC-depleted cells. RPE1 and HK2 cells were transfected with siRNAs specific to class I HDACs (HDAC1, 2, 3 and 8) and HDAC6, a class II HDAC, and cultured in serum-deprived medium for cilia formation (Figs. 8, 10). Two siRNAs for each HDAC were used (Figs. 8, 10). Approximately 70% of control cells formed cilia (Figs. 9B, 11B). The rate of cilia formation was significantly reduced in HDAC3- and HDAC8-depleted RPE1 and HK2 cells (Figs. 9B, 11B). Furthermore, the ciliary length was significantly shortened in HDAC8-depleted RPE1 and H2 cells, respectively (Figs. 9C, 11C). But, the ciliary length was slightly shortened in HDAC8-depleted HK2 cells (Fig. 11C). The proportion of the ciliary length distribution was wider than *siCTL*-treated group

(Fig 11C). These results suggest that HDAC3 and 8 are involved in cilia assembly and elongation.

Cell cycle analysis of HDAC-depleted cells

To know whether the observation that HDAC3 and 8 are involved in cilium formation and elongation is due to the effect of cell cycle arrest or the delay of cell cycle progression, I analyzed cell cycle distribution of HDAC-depleted cells. RPE1 cells were transfected with siRNAs targeting each HDAC for 24 or 48 h, and then were serum-deprived. Cells of each time point were analyzed with DNA contents (Fig. 12A). Depletion of HDAC3 increased the M phase population from 8% to 22% (Fig. 12B). Depletion of HDAC8 increased the S phase population from 15% to 27%, compared to *siCTL*-treated cells before serum starvation (Fig. 12B). No significant difference in G0 was observed in HDAC8-depleted cells (Fig. 12B). These results may indicate the effect of cell cycle arrest or the delay of cell cycle progression.

Involvement of HDAC8 in the cilium formation and elongation is not due to the effect of cell cycle arrest or the delay of cell cycle progression

To identify whether involvement of HDAC8 in the cilium assembly and elongation results from the effect of cell cycle arrest or the delay of cell cycle progression, I observed the rate of cilium assembly and measured the ciliary length in serum-starved periods. RPE1 cells were transfected with *siCTL* and siRNAs specific to HDAC8, respectively, cultured for 48 hours, then were serum-deprived for 48, 72, or 96 hours. The rate of cilium assembly and ciliary length was not increased in

HDAC8-depleted cells (Figs 13A, B). These results suggest that the implication of HDAC8 in the cilium formation and elongation is not due to the delay of cell cycle progression.

HDAC8 is required for cilia formation

Cilia formation was induced at different knockdown periods to determine at which point HDAC8 participates in cilia assembly. RPE1 cells were transfected with *siHDAC8* and cultured for 6, 24 or 48 h before transfer to serum-deprived medium. The cellular HDAC8 level was decreased gradually and was barely detected at 48 h after siRNA transfection (Fig. 14). The rate of cilia assembly and ciliary length were also reduced in a knockdown-period-dependent manner (Figs. 15B, C). These results imply that the cilia assembly rate depends on HDAC8 expression.

Rescue of the HDAC8 knockdown phenotype in cilia formation

I performed knockdown-rescue experiments to determine the importance of HDAC8 deacetylase activity in the cilium formation. The siRNA-resistant forms of wild type and deacetylase-dead HDAC8 constructs were transfected into HDAC8-depleted cells, respectively. Immunoblot analyses revealed that comparable amounts of ectopic HDAC8 protein were expressed in HDAC8-depleted RPE1 cells (Fig. 16). Cells were cultured in serum-deprived medium to induce cilia formation. Overexpression of HDAC8 increased the number of cells with cilia, although the difference did not reach statistical significance (Fig. 17B). A slight increase in ciliary length was also observed in HDAC8-overexpressing cells, although the difference

was not significant (Fig. 17C). Ectopic HDAC8 expression rescued the cilia assembly rate and ciliary length of HDAC8-depleted cells (Figs. 17B, C). However, as the deacetylase-dead HDAC8 mutant also rescued the cilium formation rate and ciliary length to a certain degree (Figs. 17B, C), HDAC8 may have a non-canonical function in cilia assembly and ciliary length control. These results suggest that the deacetylase activity of HDAC8 may not be critical for its function in cilia assembly and ciliary length control.

HDAC8 does not control proximity between mother and daughter centrioles

It was recently reported that during the early phase of ciliogenesis, Neurl-4, a daughter centriole-associated E3 Ub ligase, translocates to the mother centriole for CP110 removal (Loukil et al., 2017). This translocation requires the proximity between the mother and the daughter centrioles. If the proximity is too far for Neurl-4 to translocate and associate with the mother centriole, the removal of CP110 from the mother centriole fails, thus inhibiting the cilium assembly (Loukil et al., 2017). As HDAC8 is known to regulate the centrosome separation and MT organization in interphase cells, depletion of HDAC8 induces the centrosome splitting as a defect in MT anchoring around the centrosome (Yamauchi et al., 2011). These results propose that HDAC8 regulates the assembly and elongation of the cilium by controlling proximity between mother and daughter centriole. So, I tested whether HDAC8 is involved in centrosome splitting. RPE1 cells were transfected with *siHDAC8* and *siRootletin* and cultured for 48 h. Immunoblot analyses confirmed the specific depletion of HDAC8 and Rootletin (Fig. 18A). Centrosome splitting was observed in

Rootletin-depleted cells but not HDAC8- depleted cells (Figs. 18B, C). These results suggest that, unlike Rootletin, HDAC8 is not involved in centrosome splitting. Together with these results, HDAC8 may not control cilia assembly and elongation by controlling proximity between mother and daughter centriole.

HDAC8 is not involved in cilia disassembly

HDAC6 is essential for cilia disassembly by deacetylating α-tubulin in the microtubules of the axoneme (Ran et al., 2015). I determined whether HDAC8 is involved in cilia disassembly. RPE1 cells were transfected with siRNAs targeting HDAC8 and HDAC6. After 48 h, cells were cultured in serum-deprived medium for 48 h to induce cilium assembly. Immunoblot analyses confirmed the specific depletion of HDAC8 and HDAC6 (Fig. 19 A). Consistent with the data shown in Figs. 9B and 11B, cilia formation was significantly reduced in HDAC8-depleted cells but not in HDAC6-depleted cells (Fig. 19C). Disassembly of primary cilia initiated 4 h after transferring the cells into serum-containing medium (Fig. 19C). The number of cells with cilia was rapidly reduced in control and HDAC8-depleted cells but not in HDAC6-depleted cells (Fig. 19C). These results suggest that, unlike HDAC6, HDAC8 is not implicated in primary cilia disassembly.

Sub-ciliary localization of class Ⅰ HDACs

I performed immunostaining to determine the localization of class Ⅰ HDACs in ciliated RPE1 cells. The HDAC1, 2, and 3 were found to the nuclei and the HDAC8 was detected in the cytosol. The HDAC1 and 6 signals were specifically

detected in the basal body (Fig. 20). These observation suggest that each HDAC3, 8, and 6 do not share substrates for their biological functions.

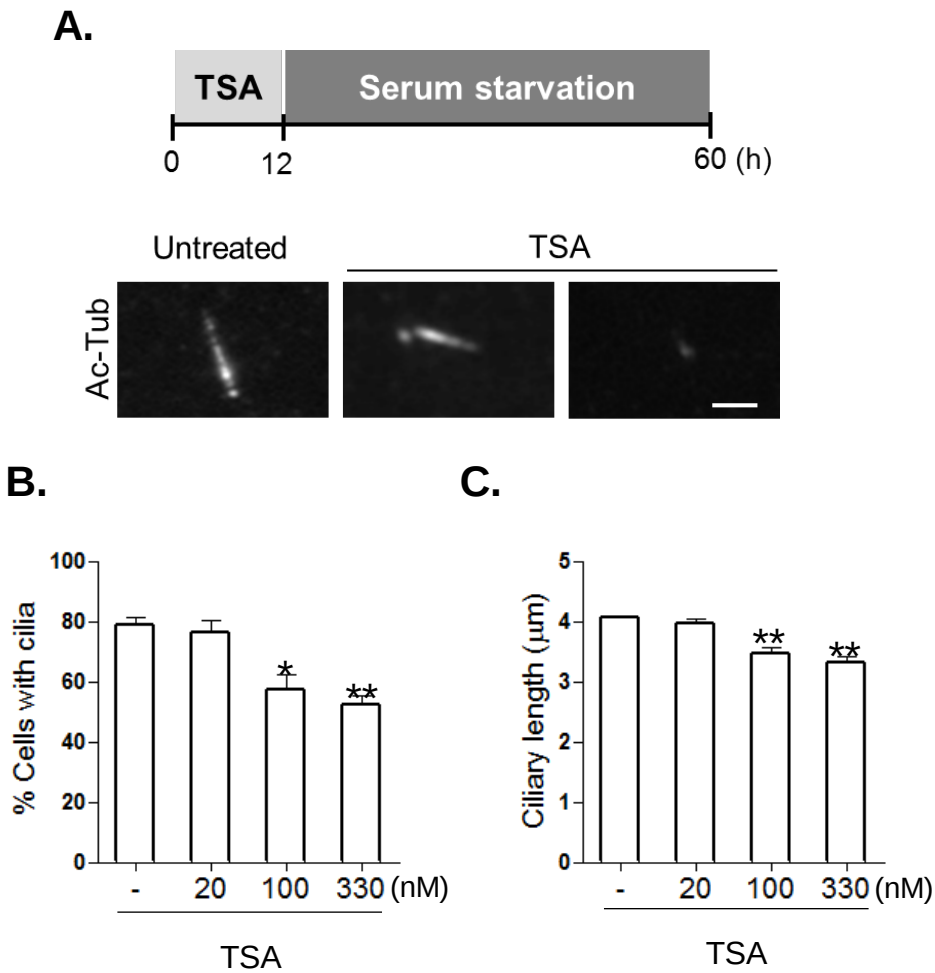


Figure 7. Effects of TSA on the cilium formation. (A) RPE1 cells were pre-treated with TSA for 12 h and cultured in serum-deprived medium for 48 h. Cells were immunostained with the acetylated α-tubulin antibody. Scale bar, 2 μm. (B) The number of cells with cilia was determined. More than 100 cells per experimental group were counted in 3 independent experiments. (C) Ciliary length was measured. More than 30 cilia per experimental group were measured in 3 independent experiments. Values are presented as the mean ± standard error. *, $P<0.05$; **, $P< 0.01$.

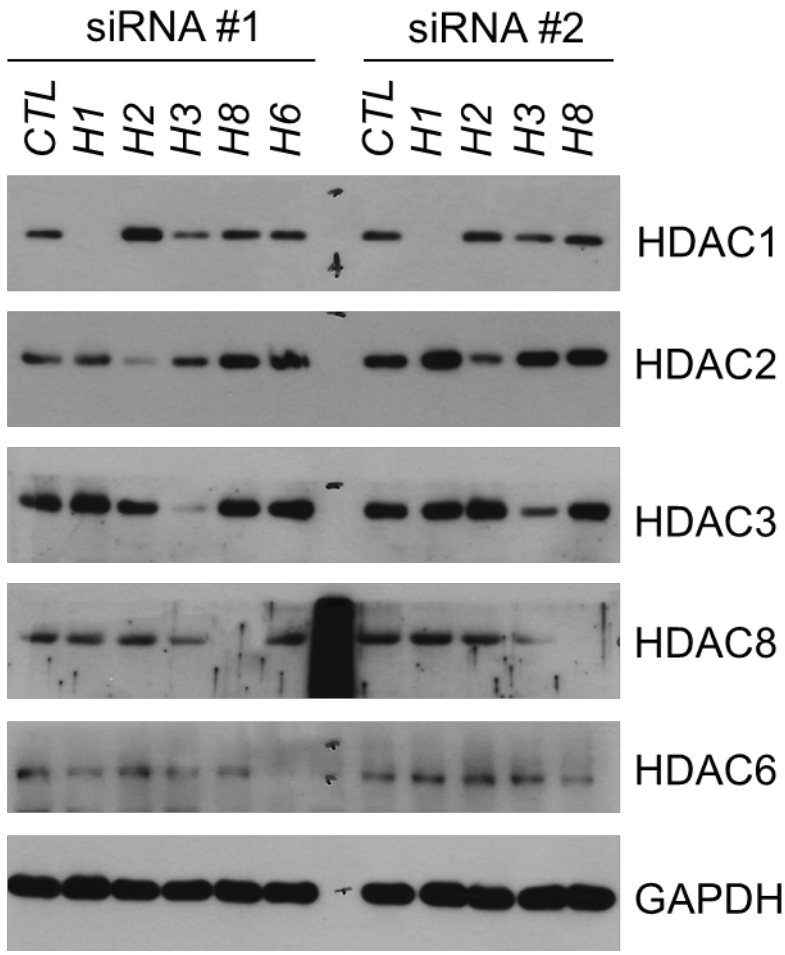


Figure 8. Depletion of endogenous class Ⅰ HDACs and HDAC6 in RPE1 cells. RPE1 cells were transfected with two siRNAs specific to *HDAC1*, *2*, *3*, *8* or *6* and cultured for 48 hours. Cell lysates were subjected to immunoblot analysis to confirm the depletion of HDACs.

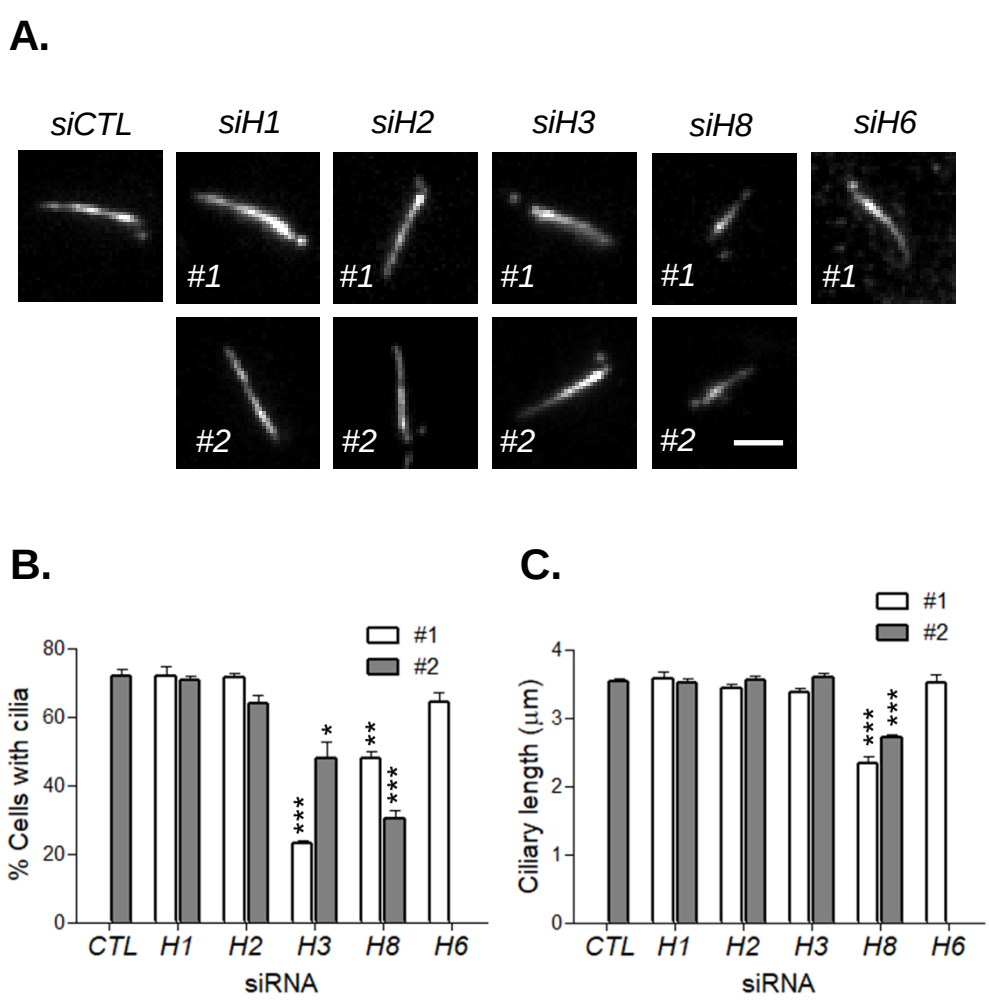


Figure 9**.** Involvement of the selected HDACs in primary cilia formation. Forty-eight hours after the siRNAs transfection, cells were cultured in serum-deprived medium for an additional 48 h. (A) Cells were immunostained with the acetylated α-tubulin antibody. Scale bar, 2 μm. (B) The number of cells with cilia was determined. More than 100 cells per group were counted in 3 independent experiments. (C) Ciliary length was measured. More than 30 cilia per experimental group were measured in 3 independent experiments. Values are presented as the mean ± standard error. *, P< 0.05; **, P< 0.01; ***, P< 0.001.

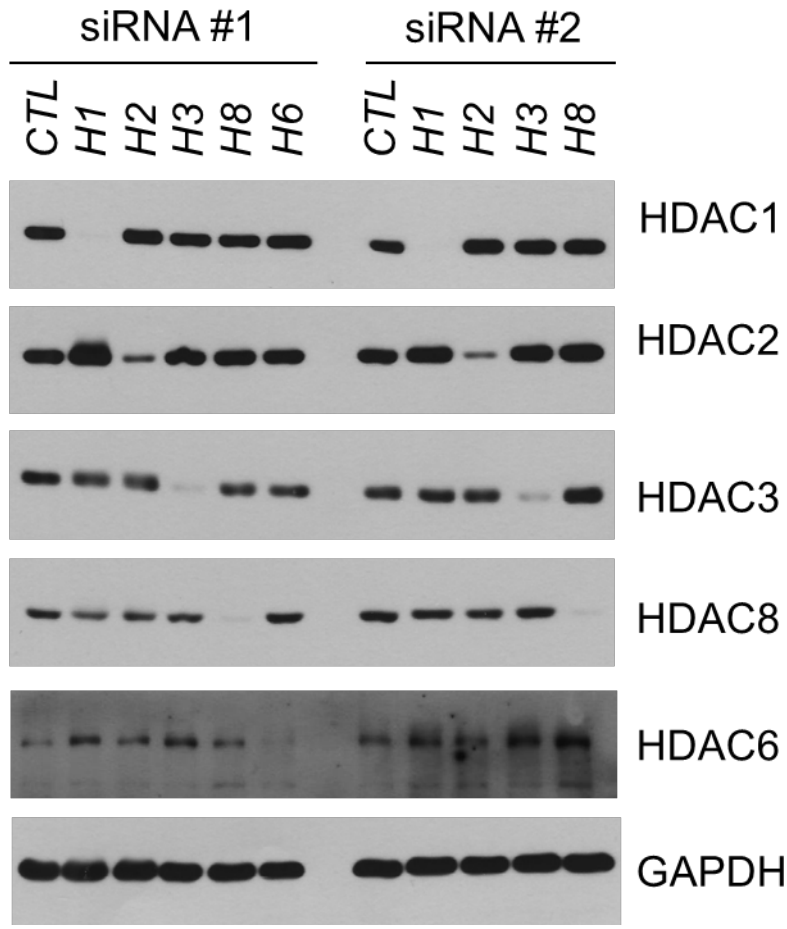


Figure 10. Depletion of endogenous class Ⅰ HDACs and HDAC6 in HK2 cells. HK2 cells were transfected with two siRNAs specific to *HDAC1, 2, 3, 8* or *6* and cultured for 48 hours. Cell lysates were subjected to immunoblot analysis to confirm the depletion of HDACs.

Figure 11**.** Involvement of the selected HDACs in primary cilia formation. Forty-eight hours after the siRNAs transfection, cells were cultured in serum-deprived medium for an additional 48 h. (A) Cells were immunostained with the acetylated α-tubulin antibody. Scale bar, 2 μm. (B) The number of cells with cilia was determined. More than 100 cells per group were counted in 3 independent experiments. (C) Ciliary length was measured. More than 30 cilia per experimental group were measured in 3 independent experiments. Values are presented as the mean ± standard error. *, $P< 0.05$; **, $P< 0.01$; ***, $P< 0.001$.

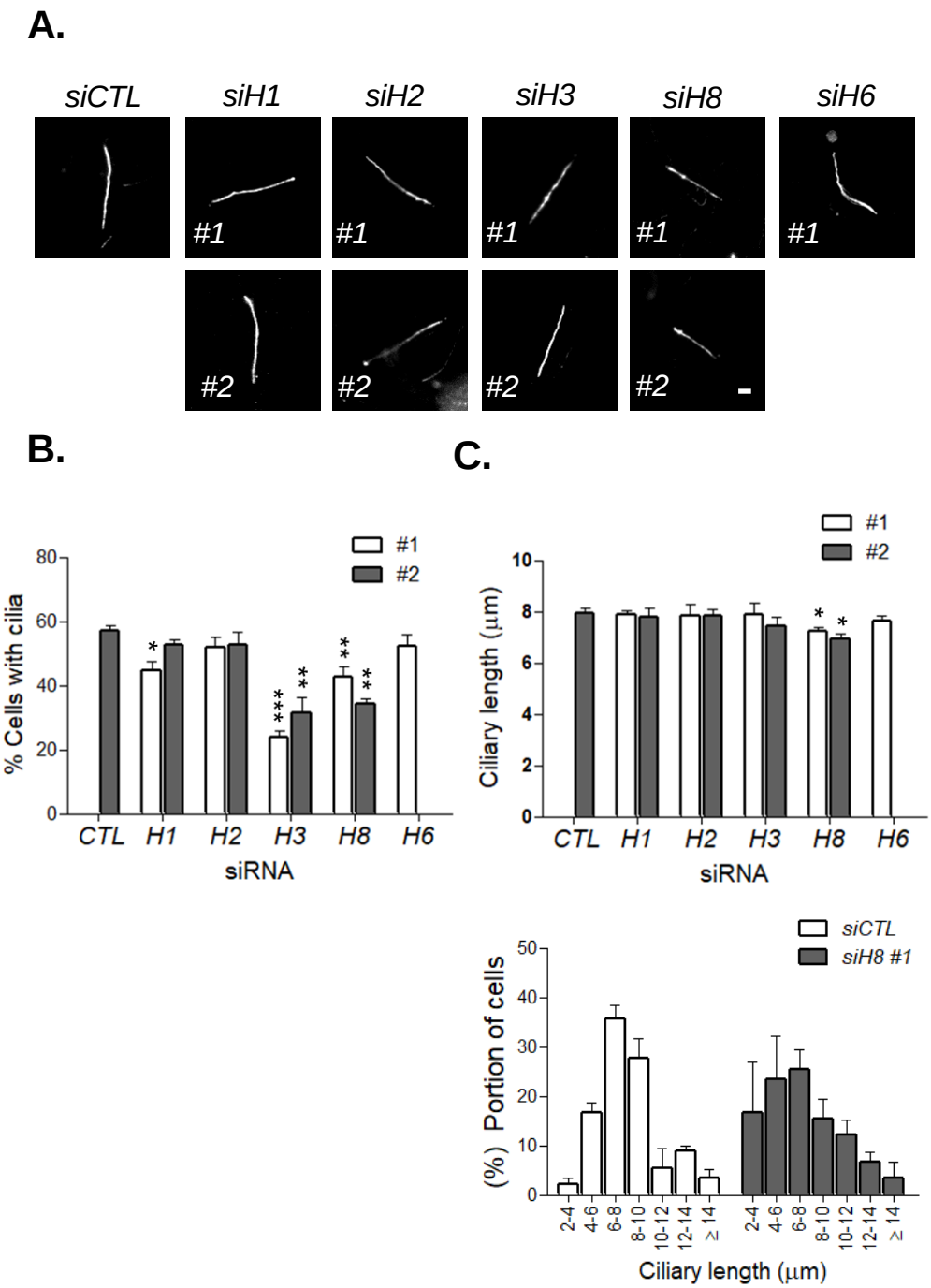
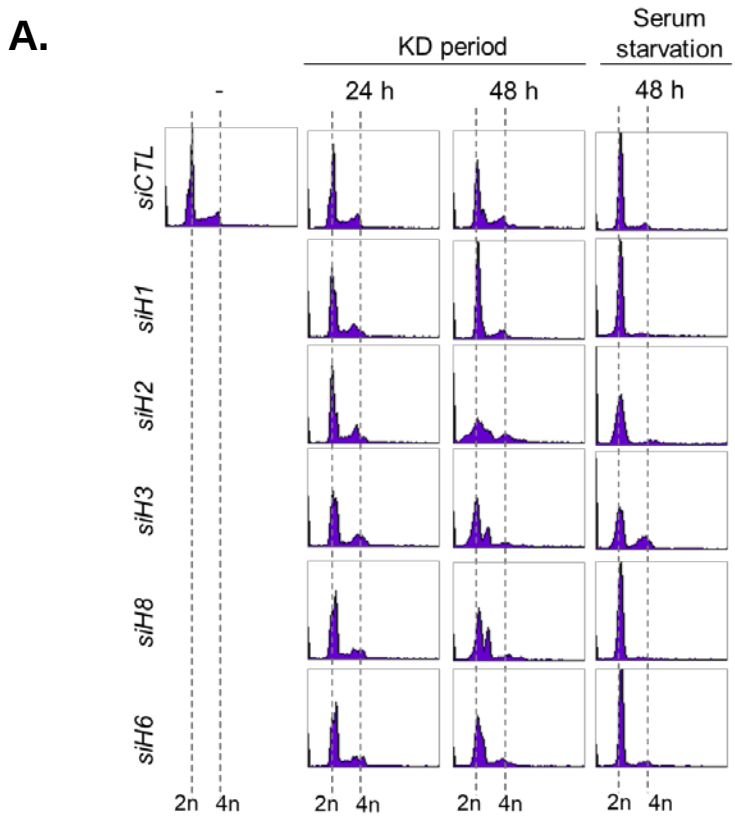
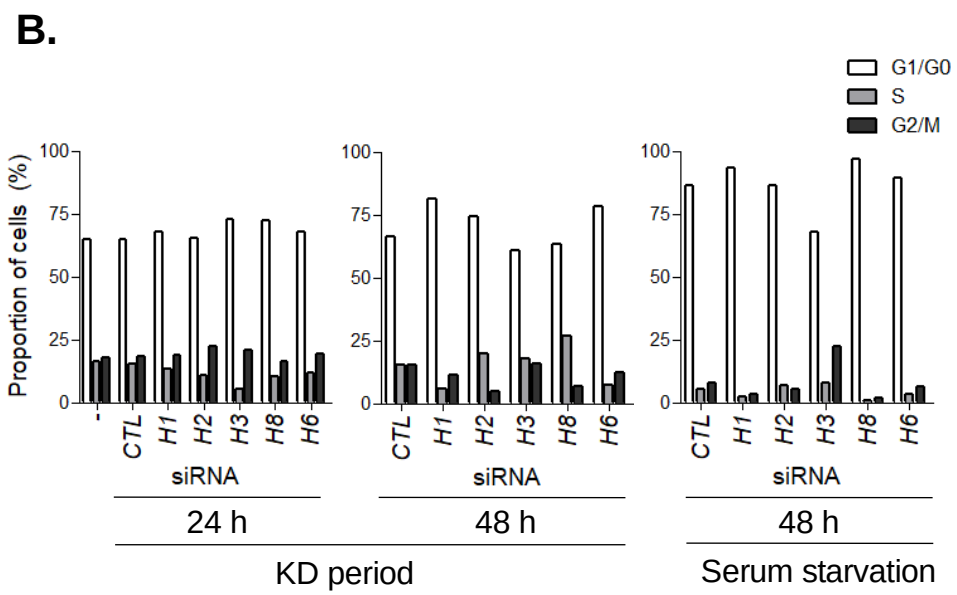

A.
siCTL
siH1
siH2
siH3
siH8
siH6
#1
#2
B.
% Cells with cilia
#1
#2
CTL
H1
H2
H3
H8
H6
siRNA
C.
Ciliary length (μm)
siCTL
siH8 #1
(%) Portion of cells
2-4
4-6
6-8
8-10
10-12
12-14
≥ 14
Ciliary length (μm)

Figure 12. Flow cytometric DNA content analysis of HDAC-depleted RPE1 cells. RPE1 cells were transfected with siRNAs specific to class Ⅰ HDACs and HDAC6 and cultured for 24 or 48 h. Cells were then cultured in serum-deprived medium for 24 or 48 h. (A) Cells were stained with propidium iodide (PI) and analysed by FACS. (B) Quantitative data shows cell cycle effects of depletion of class Ⅰ HDACs and HDAC6.


A.
KD period
Serum starvation
-
24 h
48 h
48 h
siCTL
siH1
siH2
siH3
siH8
siH6
2n 4n
2n 4n
2n 4n
2n 4n


B.
G1/G0
S
G2/M
Proportion of cells (%)
100
75
50
25
0
-
CTL
H1
H2
H3
H8
H6
siRNA
24 h
48 h
48 h
KD period
Serum starvation

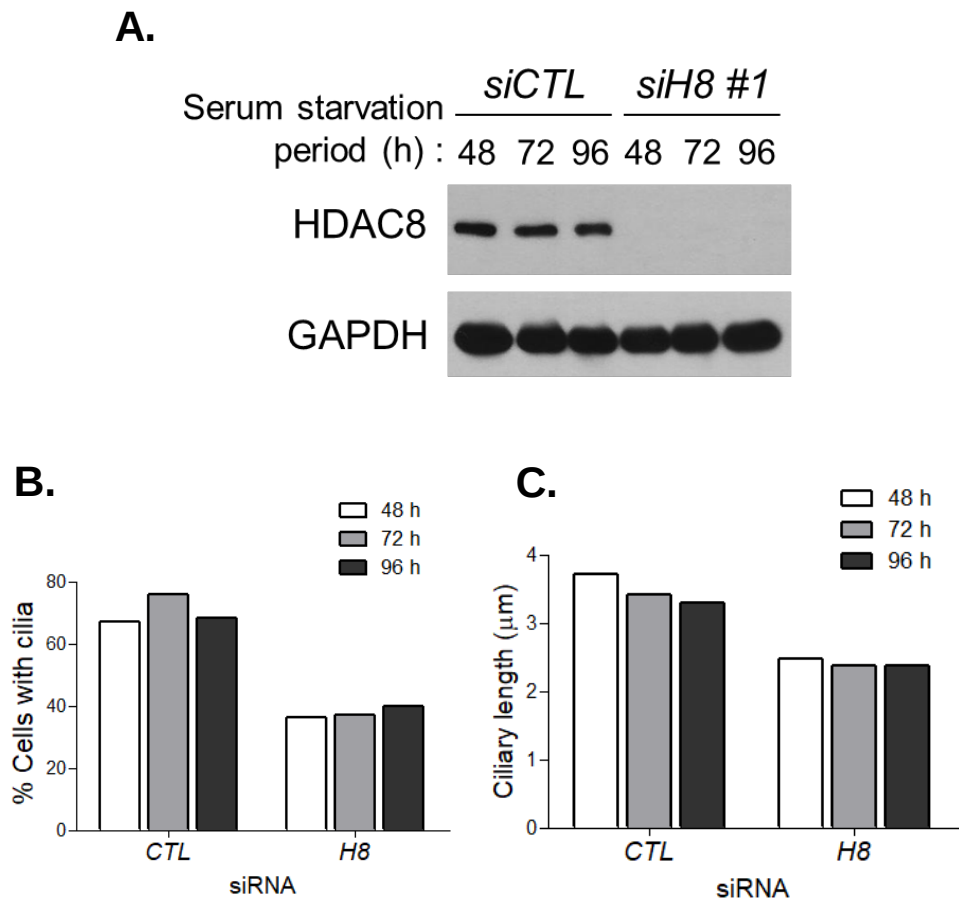


Figure 13. Involvement of HDAC8 in the cilium formation and elongation is not due to the effect of cell cycle arrest. RPE1 cells were transfected with siRNAs specific to CTL and HDAC8, cultured for 48 hours, then transferred in serum-deprived medium for 48, 72, or 96 hours. (A) The cells were subjected to immunoblot analysis with HDAC8 or GAPDH antibodies. (B) The number of cells with cilia was determined. More than 100 cells per group were counted. (C) Ciliary length was measured. More than 30 cilia were measured

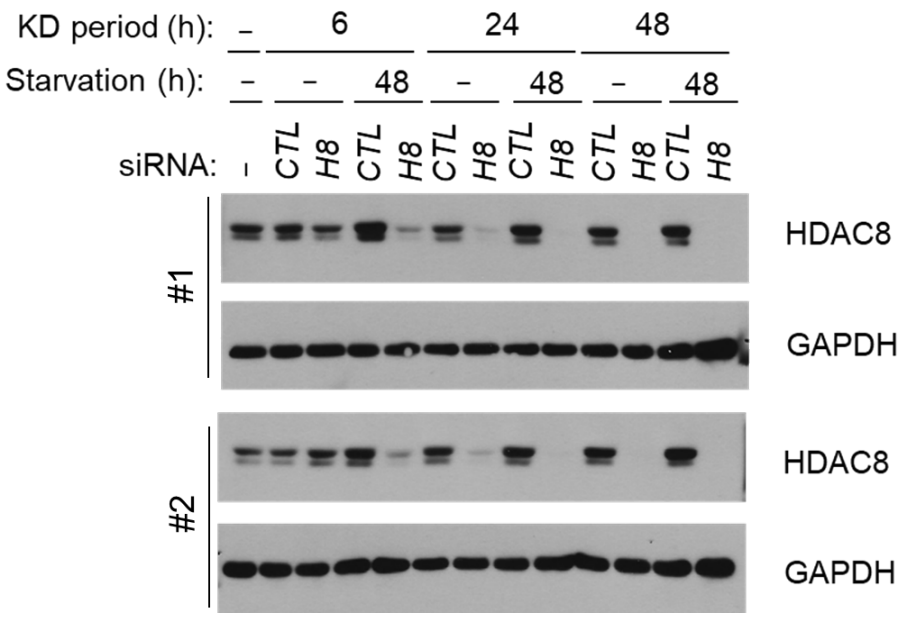
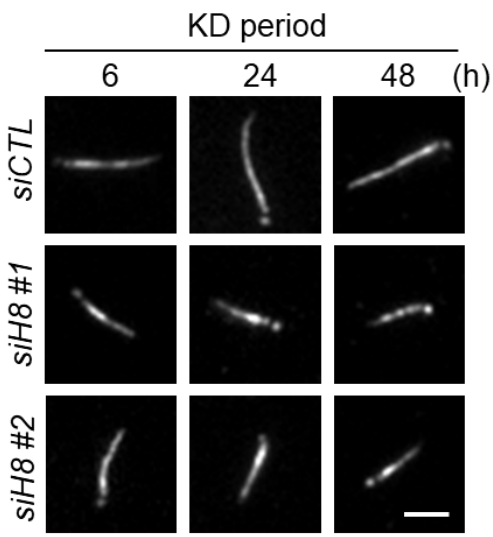


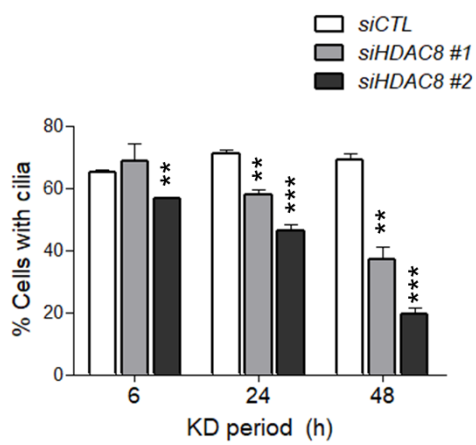
Figure 14. Depletion of endogenous HDAC8 in RPE1 cells. RPE1 cells were transfected with *siHDAC8* and cultured for 6, 24, or 48 h. Cells were then cultured in serum-deprived medium for 48 h. Cells were subjected to immunoblot analysis with antibodies specific to HDAC8 and GAPDH.

Figure 15. HDAC8 is required for primary cilia formation. (A) Cells were immunostained with the acetylated α-tubulin antibody. Scale bar, 2 μm. (B) The number of cells with cilia was determined. More than 100 cells per group were counted in 3 independent experiments. (C) Ciliary length was measured. More than 30 cilia per experimental group were measured in 3 independent experiments. Values are presented as the mean ± standard error. *, $P< 0.05$; **, $P< 0.01$; ***, $P< 0.001$.

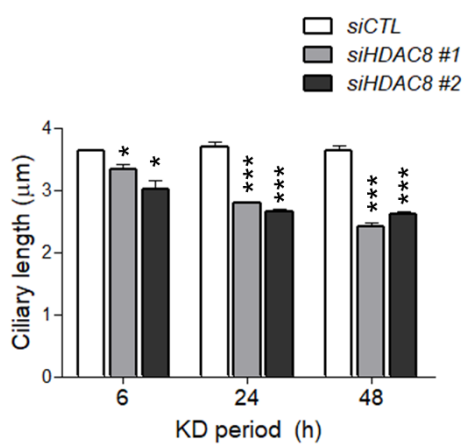
A.


KD period
6
24
48
(h)
siCTL
siH8 #1
siH8 #2

B.


siCTL
siHDAC8 #1
siHDAC8 #2
% Cells with cilia
80
60
40
20
0
**
**
***
**
***
6
24
48
KD period (h)

C.


siCTL
siHDAC8 #1
siHDAC8 #2
Ciliary length (μm)
4
3
2
1
0
*
*
***
***
***
***
6
24
48
KD period (h)

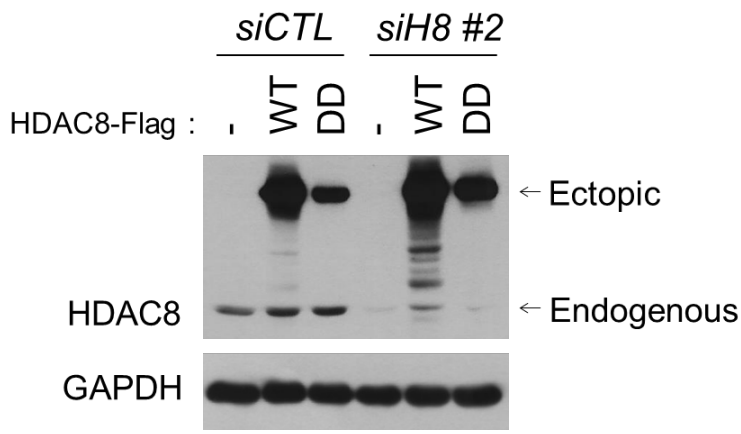


Figure 16**.** Depletion of endogenous HDAC8 and ectopic expression of HDAC8 in RPE1 cells. RPE1 cells were transfected with *siCTL* or *siHDAC8*. Six hours later, cells were transfected with expression vectors for RNAi-resistant forms of wild type (WT) and deacetylase-dead (DD) HDAC8, cultured for 42 h and transferred to serum-deprived medium for 48 h. Cells were subjected to immunoblot analysis with the indicated antibodies

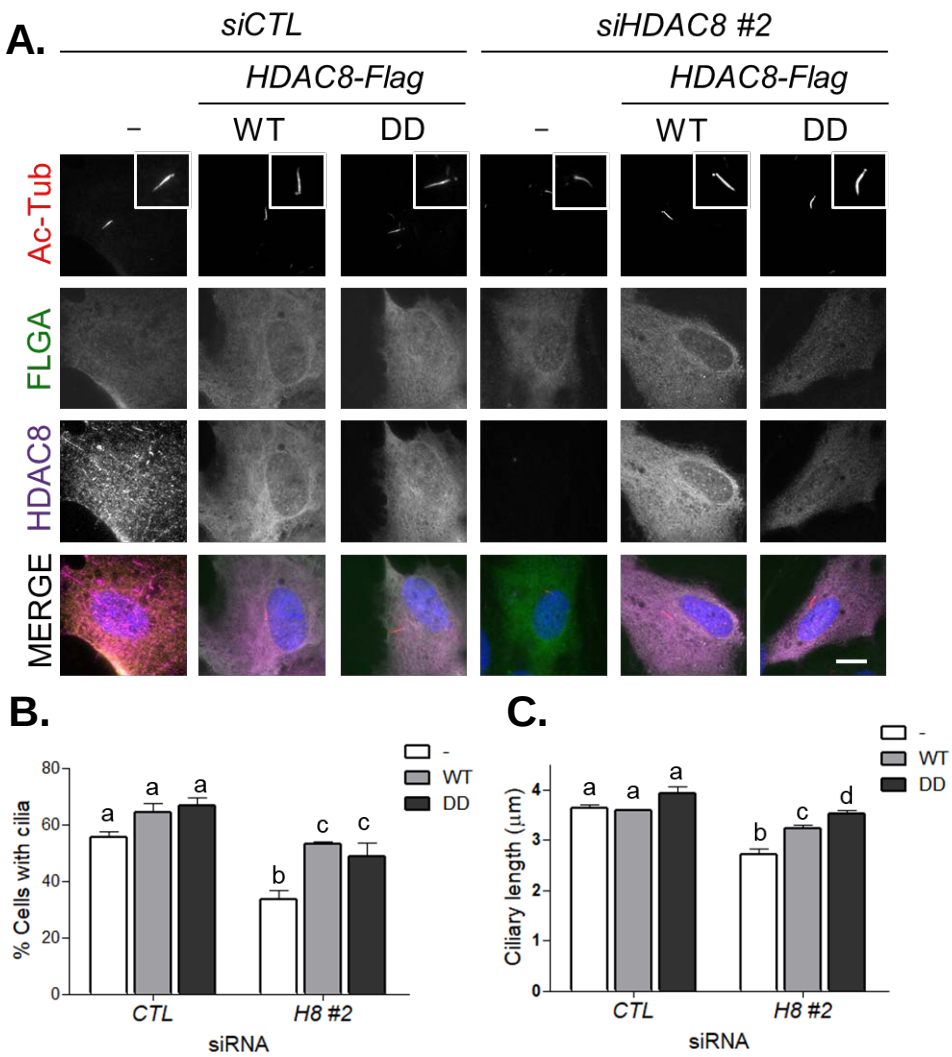
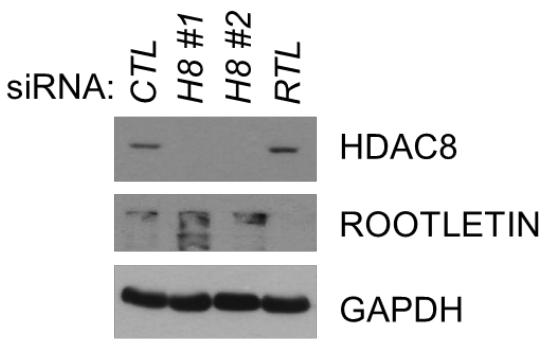


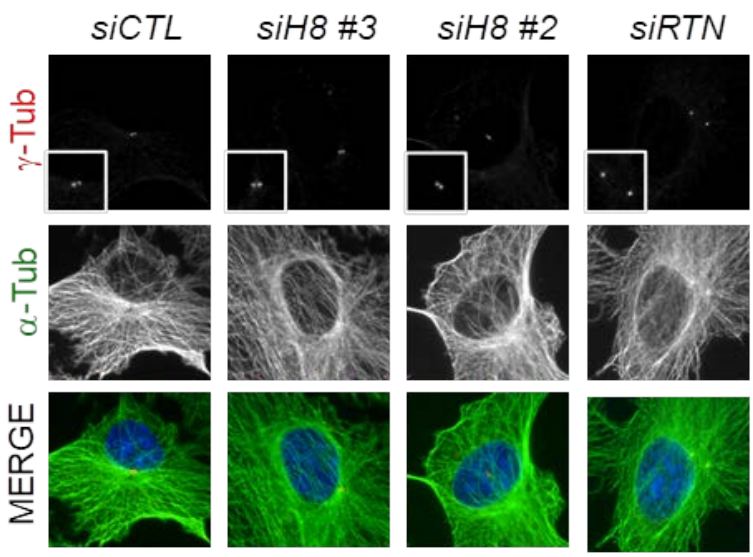
Figure 17. HDAC8 deacetylase activity is not required for the cilium formation and elongation. (A) Cells were co-immunostained with antibodies specific to acetylated α-tubulin, Flag, or HDAC8. Scale bar, 10 µm. (B) The number of cells with cilia was determined. More than 100 cells per group were counted in 3 independent experiments. (C) Ciliary length was measured. More than 30 cilia per experimental group were measured in 3 independent experiments. Statistical significance was analysed using two-way ANOVA and is indicated by different letters ($P<0.05$).

Figure 18. HDAC8 is not required for centrosome splitting. Growing RPE1 cells were transfected with *siHDAC8* or *siRootletin* and cultured for 48 hours (A) Cells were subjected to immunoblot analysis with antibodies specific to HDAC8, Rootletin or GAPDH. (B) Cells were co-immunostained with antibodies specific to acetylated α-tubulin and γ-tubulin. Scale bar, 10 μm. (C) Distance between two centrosomes was measured. More than 50 cells per group were counted.

A.


siRNA:
CTL
H8 #1
H8 #2
RTL
HDAC8
ROOTLETIN
GAPDH

B.


siCTL
siH8 #3
siH8 #2
siRTN
γ-Tub
α-Tub
MERGE

C.

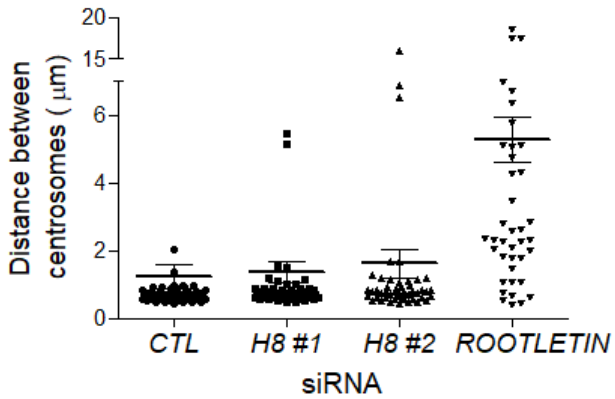
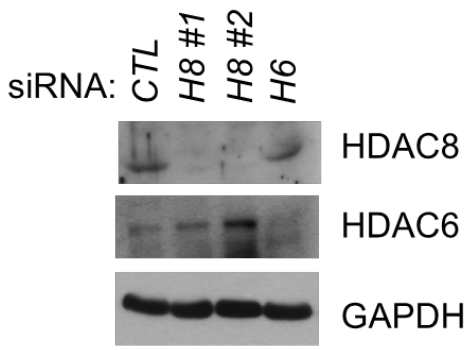
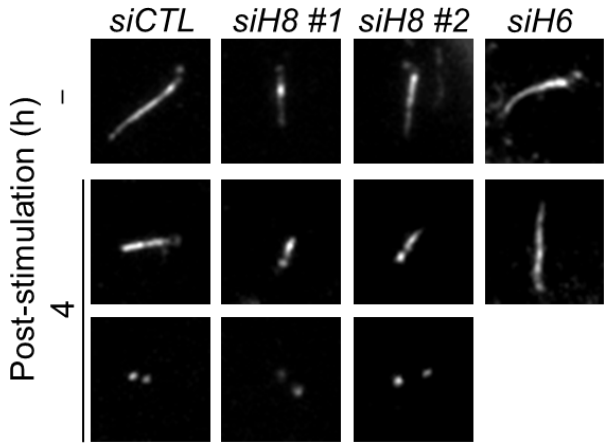

Distance between centrosomes (μm)
20
15
6
4
2
0
CTL
H8 #1
H8 #2
ROOTLETIN
siRNA

Figure 19 HDAC8 is not involved in cilium disassembly. (A) *siHDAC*-transfected RPE1 cells were cultured in serum-deprived medium for 48 h and transferred to serum-containing medium for 4 h. Cells were subjected to immunoblot analysis with antibodies specific to HDAC8, HDAC6 or GAPDH. (B, C) The number of cells with cilia was determined. More than 100 cells per group were counted in 3 independent experiments. Values are presented as the mean ± standard error. Statistical significance was analysed using two-way ANOVA and is indicated by different letters ($P<0.05$).

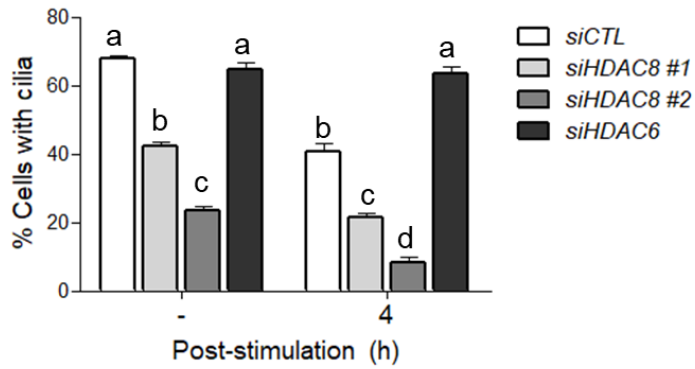
A.


siRNA: CTL H8 #1 H8 #2 H6
HDAC8
HDAC6
GAPDH

B.


siCTL
siH8 #1
siH8 #2
siH6
Post-stimulation (h)
-
4

C.


% Cells with cilia
80
60
40
20
0
a
b
c
a
b
c
d
a
-
4
Post-stimulation (h)
siCTL
siHDAC8 #1
siHDAC8 #2
siHDAC6

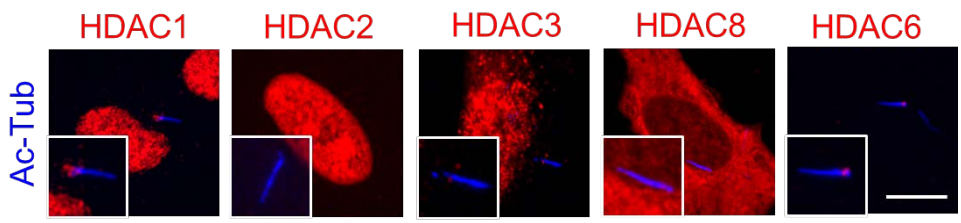


Figure 20. Localization of class 1 HDACs in ciliated RPE1 cells. RPE1 cells were cultured in serum-deprived medium for 48 hours and co-immunostained with antibodies specific to class Ⅰ HDACS (red), or acetylated a-tubulin (blue). Scale bar, 10 μm.

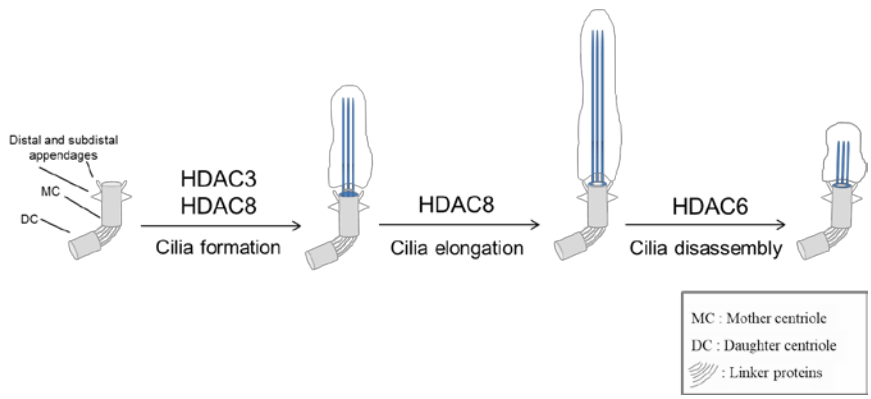


Figure 21. Model of HDAC functions in ciliogenesis. Each HDAC participates in the cilium assembly (HDAC3 and HDAC8), ciliary length (HDAC8), and the cilium disassembly (HDAC6).

Discussion

In this work, I identified deacetylases that are involved in primary cilium assembly and elongation in serum-deprived RPE1 and HK2 cells. The rate of cilium assembly was significantly reduced in HDAC3- and 8-depleted cells. Ciliary length was also reduced in HDAC8-depleted cells. This is the first study to report that deacetylases are required for the assembly and elongation of the primary cilium.

When cells are cultured in serum-deprived medium, the number of assembled cilia continuously increases in cells entering G0/G1 phase (Sanchez and Dynlacht, 2016). My knockdown experiments revealed a positive correlation between the cilium assembly rate and the cellular level of HDAC8, suggesting that HDAC8 is involved in primary cilium assembly (Figs. 8-11). This result is in contrast to HDAC6, which plays a role in cilium disassembly (Pugacheva et al., 2007). In fact, the cilium formation rate was inhibited in HDAC8-depleted cells but not in HDAC6-depleted cells (Figs. 9, 11). Furthermore, cilium disassembly was suppressed in HDAC6-depleted cells but not in HDAC8-depleted cells (Fig. 19). Based on these results, I conclude that the target proteins of HDAC8 for primary cilium assembly differ from those of HDAC6 for primary cilium disassembly.

The shortening of ciliary length differs between HDAC8-depledted RPE1 and HK2 cells. In RPE1 cells, depletion of HDAC8 shortened about 30% ciliary length, but in HK2 cells, it slightly but significantly was shortened and a proportion of ciliary length distribution was wider than in control cells. It implies that the regulatory mechanism of ciliary length between RPE1 and HK2 cells may be different each other.

The ciliary length of RPE1 and HK2 cells is about 3.6 μm and 8 μm, respectively. This may make the regulation of ciliary length different such as transport of ciliary length regulators.

HDAC8 is involved in the cilium formation and elongation in deacetylase activity-independent manner. HDACs often function in deacetylation-independent manner. For example, HDAC7 associates with Runx2 and represses its transcriptional activity in a deacetylase-independent manner (Jensen et al., 2008). Therefore, HDAC8 may regulate the cilium assembly and elongation through interaction with other proteins that control primary cilia formation and elongation.

It was recently reported that HDAC1, HDAC5 and SIRT1 have activities to suppress centrosome amplification. The abilities are associated with their capacities to localize to the centrosome (Ling et al., 2012). But, there are exceptions to this concept that HDAC3 and HDAC8 do not localize to the centrosome, but they suppressed centrosome amplification (Ling et al., 2012). HDAC8 has been implicated in centrosome splitting, an initial event of centrosome duplication (Yamauchi et al., 2011). Because the process of centrosome duplication is linked with the cell cycle, non-centrosomal events such as the mitogenic signalling are crucial for centrosome duplication. Thus, HDAC3 and HDAC8 likely may target non-centrosomal events. However, when duration of forming the cilium was increased to 96 hours, the ciliary length was not lengthened (Fig. 13). This suggests that the implication of HDAC8 in the cilium formation and elongation is at least not due to the delay of cell cycle progression.

It was previously shown that Golgi matrix protein, giantin, is required for ciliogenesis by controlling the localization of dynein-2. Depletion of giantin induces reduction in cilium formation and a failure in localization of WDR34, the intermediate chain subunit of dynein-2, in pericentriolar material (Asante et al., 2013). It is independent of the Rab11-Rabin8-Rab8 pathway. The localization of giantin in the juxtanuclear region is regulated by HDAC8. Depletion of HDAC8 causes the dispersal of giantin throughout the cytoplasm (Yamauchi et al., 2011). It is possible that HDAC8 may be involved in cilia formation by controlling the juxtanuclear localization of giantin. However, depletion of giantin causes an increase in ciliary length. This differs from my observation that HDAC8 depletion shortened the ciliary length. Therefore, I supposed that HDAC8 contributes to ciliary length control by different mechanisms.

Primary cilium growth occurs exclusively at the plus ends of microtubules (Sung and Leroux, 2013). The building blocks of the ciliary axoneme are transported by IFT proteins (Ishikawa and Marshall, 2017). Multiple layers of regulation have been reported for ciliary length control, including transcription, ubiquitination and phosphorylation (Keeling et al., 2016). In this work, I add that a deacetylase is also involved in ciliary length control through physical interaction with regulatory factors.

It is currently understood that multiple deacetylases are involved not only in cilium disassembly but also in cilium assembly and elongation. My work describes additional mechanisms that may be involved in the regulation of cilia formation under serum-deprived conditions. I am currently investigating their mechanism of action.

Chapter 2.
Identification and functional analysis of E3 ubiquitin ligases in the centrosome

Abstract

The centrosome is assembled and segregated in tight link to the cell cycle. In non-dividing cells, the centrosome serves as a basal body for the cilium formation. The centrosome organizes the cytoplasmic MTs in interphase cells and emanates mitotic spindles during mitosis. To carry out timely functions of the centrosome, centrosomal proteins should be promptly degraded possibly by specific E3 ubiquitin ligases. Here, I identified E3 ubiquitin ligases located in the centrosome. Among 226 E3 ligases examined, I found that 31 were located at the centrosome. Furthermore, I identified that FBXO31 is involved in the suppression of centrosome amplification. On the other hand, ANAPC11, FBXL14, FBXO4, HERC3, KLHL2, PELI1, RNF135, SPSB4, and TCEB2 appeared to be implicated in the promotion of centrosome amplification.

Introduction

The centrosomes play diverse roles during the cell cycle. In interphase, the centrosome organizes the cytoplasmic MTs to regulate cell shape, migration, polarity, and support of cytoskeleton and during the mitosis assembles mitotic bipolar spindle to segregate duplicated chromosomes into daughter cells (Bettencourt-Dias and Glover, 2007).

Centrosome duplication occurs once per the cell cycle. The failure in regulating centrosome number increases chromosomal instability, thus leads to tumor formation, which have been observed in various cancer cells (Godinho and Pellman, 2014). Thus, the number of centrosomes in a cell should be thoroughly regulated. And the regulatory mechanisms control centrosome duplication via diverse posttranslational modifications.

Protein phosphorylation is an important posttranslational modification in regulating centrosome duplication. PLK4 is a master regulator for centrosome duplication. PLK4 recruits and phosphorylates centriole assembly components around the proximal end of the mother centriole to assemble a new procentriole (Kim et al., 2013; Ohta et al., 2014). Manipulations of cellular PLK4 level by overexpression or knockdown result in increase or decrease in centrosome duplication (Bettencourt-Dias et al., 2005; Kleylein-Sohn et al., 2007). Thus, cellular PLK4 level during a procentriole assembly should be strictly controlled.

Protein ubiquitination is another posttranslational modification that regulates centrosome duplication. Specific centrosomal proteins undergo the process

of drastic synthesis or degradation during the cell cycle and carry out own functions. Indeed, it was previous shown that treatment of MG132 increased amount of PLK4 and induced centrosome overduplication (Guderian et al., 2010). Ubiquitination of PLK4 is important for regulating PLK4 stability and thus the centriole number. The auto-phosphorylation of PLK4 affects the centriole number by controlling the degradation mediated by FBXW11/β-TrCP (Guderian et al., 2010). Activity of Cullin1 (CUL1) is essential for the degradation of PLK4. The degradation of PLK4 by CUL1 induces the overexpression of cyclin E/CDK2, and regulates the stability of basal PLK4 (Korzeniewski et al., 2009). Mind bomb 1 (MIB1) binds to and ubiquitinates PLK4. Ubiquitinated PLK4 is degraded and rarely detected in the centrosome. This modification regulates the capacity to interact with PLK4 and (an) other centrosomal protein(s) and results in the inhibition of centriole over-duplication (Wang et al., 2016). SAS6 is another centrosomal target for proteasomal degradation. Ubiquitination of SAS6 by FBXW5 is important for regulating of centrosome number (Puklowski et al., 2011).

Centrosomal E3 ubiquitin ligases that degrade centrosomal proteins during the cell cycle are important for proper progression of centrosome duplication. Despite the importance of the PTMs in controlling centrosome duplication, little studies have shown the functions and mechanisms of some E3 ubiquitin ligases in the centrosome duplication. Therefore, there might be more centrosomal E3 Ub ligases for controlling centrosome number.

So far, about 600 E3 ubiquitin ligases have been found (Li et al., 2008). In this work, I identified E3 ubiquitin ligases located in the centrosome among 226 E3

ligases, a gift from professor Ji, U. of Ajou, and examined involvement of the E3 ligases in the suppression of centrosome amplification. I found that 31 are located in the centrosome and of the identified 31 E3 ligases, FBXO31 is involved in the suppression of centrosome amplification and ANAPC11, FBXL14, FBXO4, HERC3, KLHL2, PELI1, RNF135, SPSB4, and TCEB2 promoted centrosome amplification are implicated in the promotion of centrosome amplification.

Materials and Methods

Cell culture and drug treatment

U2OS cells were cultured in McCoy'5A (Welgene, LM 005-01) supplemented with 10% foetal bovine serum (FBS) (Welgene, S101-01) and Plasmocin™ (Invivogen, anti-mpt). For centrosome amplification assay, 2 mM of HU was added to growing U2OS cells for 72 h.

Constructs and transfection of plasmids

E3 ubiquitin ligase plasmids subcloned into the *pDEST53(GFP)* vector were gifted from professor Jhi, U. of Ajou. *pLVX-IRES-Puro* GFP vector was used as negative control. Plasmids were transfected into U2OS cells with Fugene HD (Promega, E2311).

Antibodies

The primary antibodies used were specific to Centrin-2 (Millipore, 04-1624), GFP (Santacruz; sc-9996, abcam; ab6556), γ-tubulin (Santacruz, sc-7396), or GAPDH (Ambion, AM4300). The secondary antibodies used were conjugated to fluorescent dyes (Alexa-488, Alexa-555, Alexa-594, and Alexa-647; Life Technologies) or hydrogen peroxidase (mouse, Sigma; rabbit, Millipore).

Immunoblot analysis

Cells were lysed with 1x SDS sample buffer and boiled for 10 min. Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with blocking buffer (5% skim milk in 0.1% Tween 20 in TBS) for 1 hour, incubated with the indicated primary antibody for 16 hours, washed four times with TBST (0.1% Tween 20 in TBS), incubated with the appropriate secondary antibody in blocking buffer for 40 min and washed four additional times with TBST. Hydrogen peroxidase signals were detected using ECL solution (ABfrontier, LF-QC0101).

Immunofluorescence microscopy

Cells grown on 12-mm coverslips were fixed with cold methanol on ice for 10 min, and permeabilized with PBST (0.3% Triton X-100 in PBS) for 10 min. Cells were blocked with blocking buffer (3% bovine serum albumin and 0.3% Triton X-100 in PBS) for 20 min, incubated with the indicated primary antibody for 1 hour, washed three times with PBST, incubated with the indicated secondary antibody for 30 min, washed three additional times with PBST, counterstained with DAPI solution for 1 min, and mounted onto glass slides with ProLong Gold antifade reagent (Invitrogen, P36930). Cells were observed under a fluorescence microscope (Olympus IX51) with a 60×/1.25 oil Iris (UFlanFl) objective lens. Images were acquired on a CCD camera (Qicam fast 1394, Qimaging) and analysed using ImagePro 5.0 (Media Cybernetics, Inc.).

Statistical analysis

All experiments were repeated at least three times. GraphPad Prism 5 was used for the unpaired two-tailed *t*-test.

Results

Identification of centrosomal localization of known E3 ubiquitin ligases in the centrosome

Ubiquitination reactions mainly have progressed in the cytosol and nucleus. But the role of E3 ligases is not fully described in the centrosome yet. And some proteins whose localizations vary are difficult to detect centrosomal localization. So, I tried to immunocytochemically detect centrosomal localization of known E3 ligases, CUL1, FBXW11, and MIB1, in the centrosome. U2OS cells were transfected with E3 ligase constructs and cultured for 24 hours. Immunoblot analyses confirmed the expressions of these E3 ligases. Centrosomal signals were detected in GFP-positive cells transfected with CUL1 and FBXW11 constructs, respectively. These results suggest that my immunocytochemical analysis is sensitive enough to identify the centrosomal localization of E3 ligases.

Identification of E3 ubiquitin ligases located in the centrosome

I identified centrosomal localization of E3 ligases among 226 E3 ligases in E3 ligase-expressing cells. U2OS cells were transfected with each E3 ligase construct, and cultured for 24 hours. Immunoblot analyses confirmed the expression of each E3 ligase (Fig. 23). ANAPC11, ASB3, ASB9, CUL1, ENC1, FBXL14, FBXL18, FBXO21, FBXO31, FBXO32, FBXO4, FBXO9, FBXW11, FBXW12, HECTD2, HERC3, KLHL2, PCGF5, PELI1, RHOBTB3, RNF125, RNF135, RNF25, SPSB1, SPSB4, TCEB2, TRIM24, TRIM36, UBE3C, VHL, and WWP2 were found at the

centrosome. The representative images and lists were shown in Fig. 24 and table 1. Of the total 31 identified E3 Ub ligases, 23 E3 ligases are novel centrosomal E3 ligases. These observations suggest that 31 E3 ligases take part in the regulation of centrosome duplication.

Functional analysis of the identified E3 ubiquitin ligases in the regulation of centrosome duplication

To obtain a clue into the roles of the identified centrosomal E3 ligases in centrosome duplication, I tested whether their overexpression regulates centrosome amplification. U2OS cells were transfected with each E3 ligases, cultured for 16 hours and treated with 2 mM of HU for 72 hours. The cells were immunostaining with GFP and Centrin-2 antibodies. In the control experiment, 30% of the cells had 5 or more Centrin-2 dots and the number was reduced to 13% in FBXO31-expressing cells, whereas the number increased the cells overexpressing ANAPC11, FBXL14, FBXO4, HERC3, KLHL2, PELI1, RNF135, SPSB4, and TCEB2, respectively. These results suggest that these E3 ubiquitin ligases are required for the regulation of centrosome duplication.

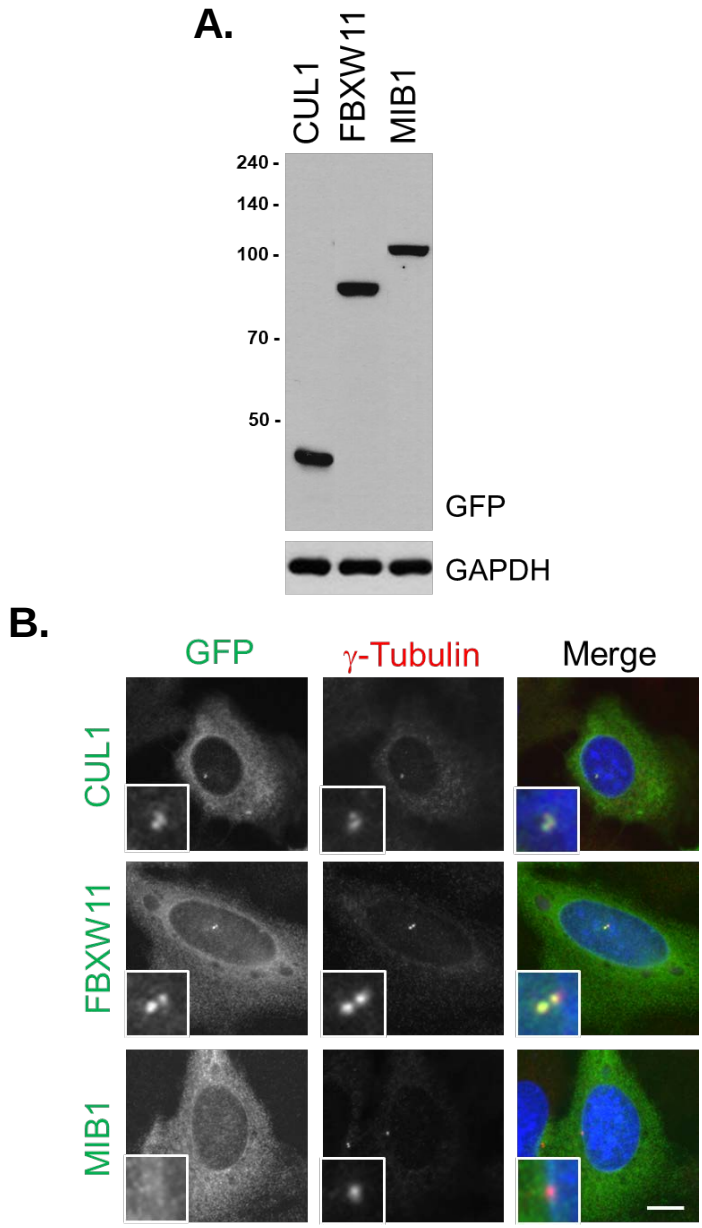


Figure 22**.** Identification of known E3 ubiquitin ligases located in the centrosome. U2OS cells were transfected with expression vectors for CUL1, FBXW11, and MIB1 and cultured for 24 hours. (A) Cells were subjected to immunoblot analysis with GFP or GAPDH antibodies. (B) Cells were co-immunostained with antibodies specific to GFP and γ-tubulin. Scale bar, 10 μm.

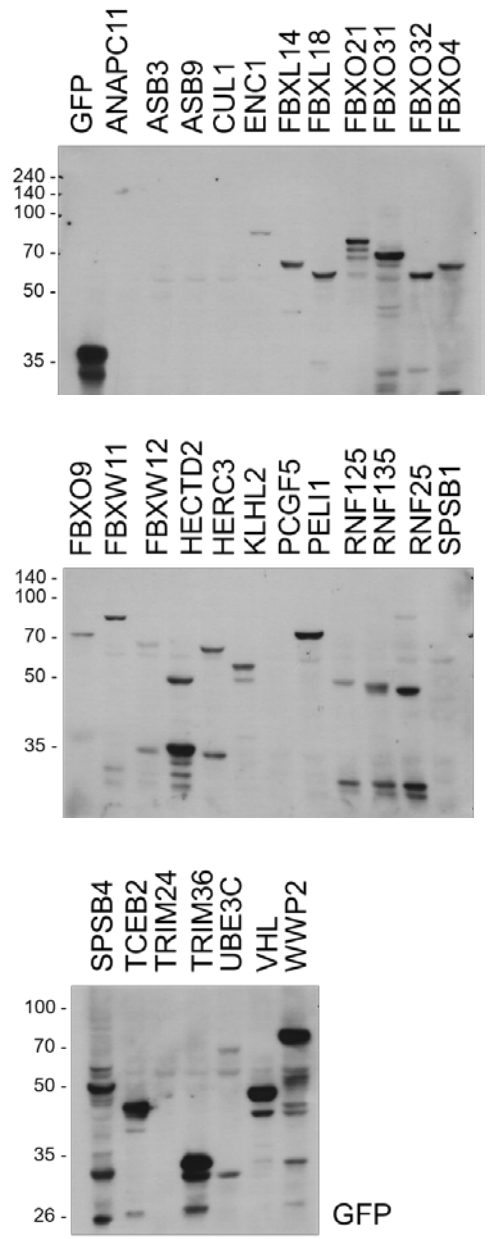


Figure 23. Expression of the E3 ubiquitin ligases. U2OS cells were transfected with the indicated expression vectors for 24 hours. Cells were subjected to immunoblot analysis with GFP antibody.

Figure 24. Identification of E3 ubiquitin ligases located in the centrosome. U2OS cells were transfected with expression vector for E3 ubiquitin ligases and cultured for 24 hours. The cells were co-immunostained with antibodies specific to GFP and γ-tubulin. Scale bar, 10 μm.

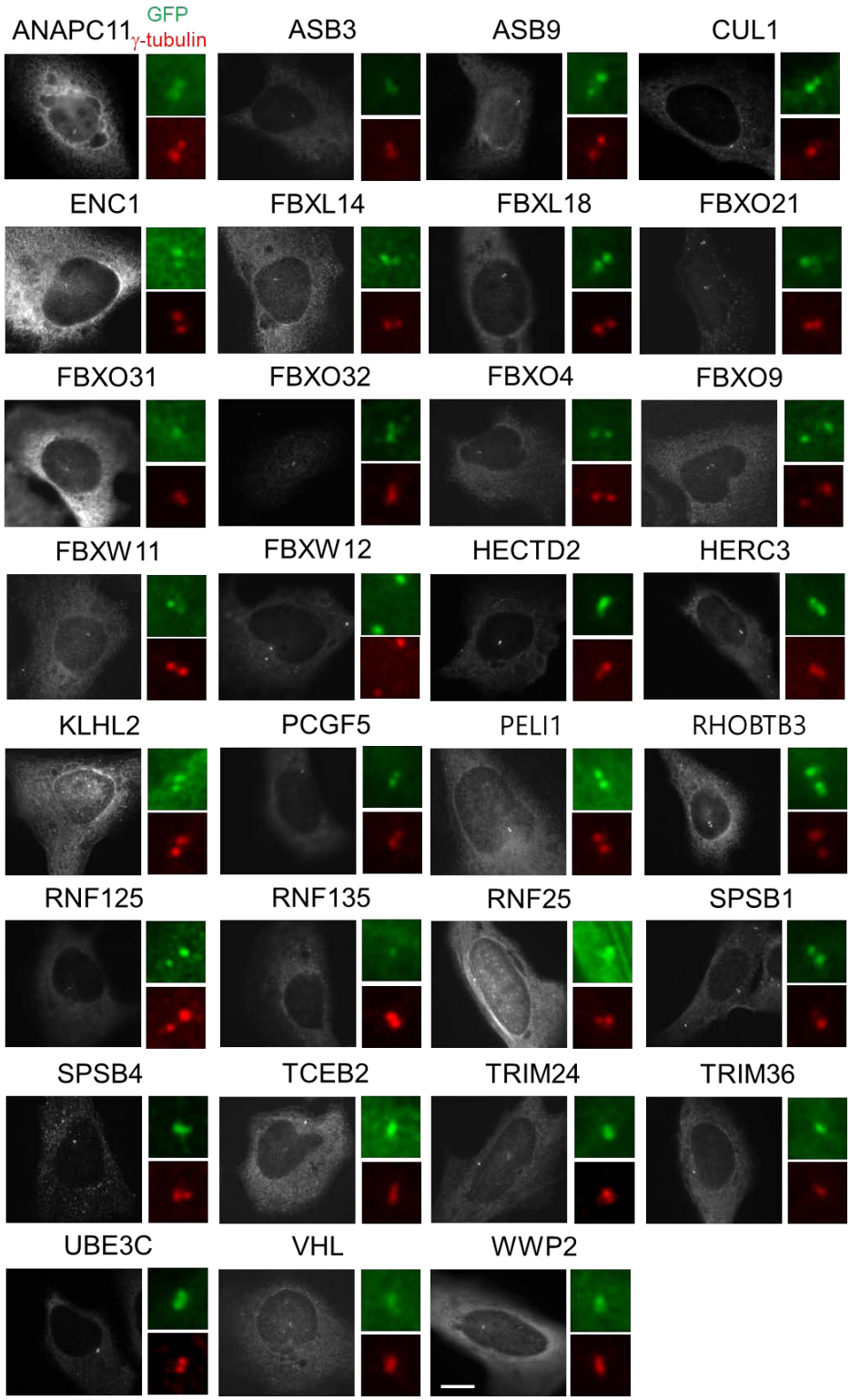
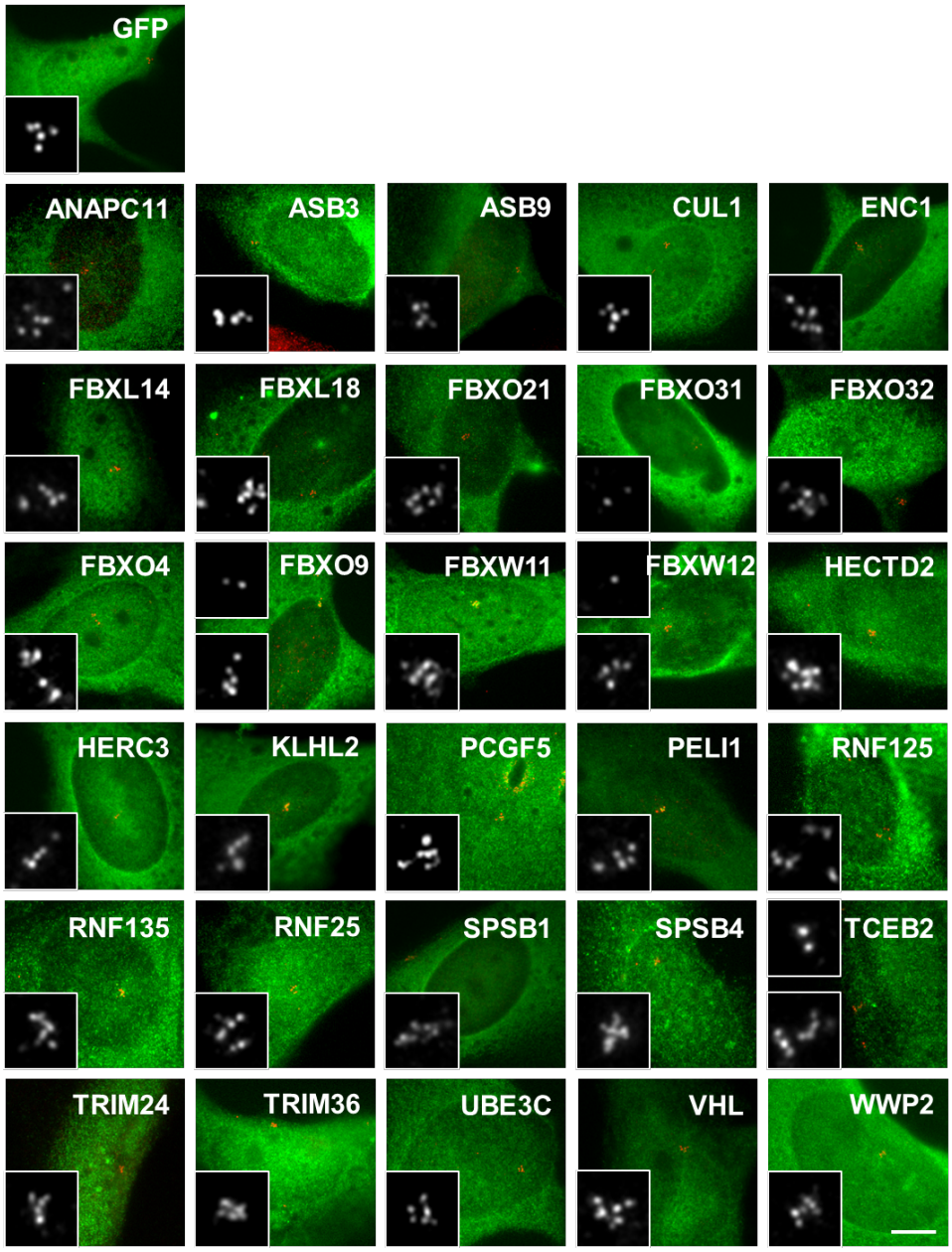

GFP
γ-tubulin
ANAPC11
ASB3
ASB9
CUL1
ENC1
FBXL14
FBXL18
FBXO21
FBXO31
FBXO32
FBXO4
FBXO9
FBXW11
FBXW12
HECTD2
HERC3
KLHL2
PCGF5
PELI1
RHOBTB3
RNF125
RNF135
RNF25
SPSB1
SPSB4
TCEB2
TRIM24
TRIM36
UBE3C
VHL
WWP2

Table 1. List of the E3 ubiquitin ligases which were detected at the centrosome.

	Genes	Known localization	Centrosomal localization
1	*ANAPC11*	N	Novel
2	*ASB3*	N, C	Novel
3	*ASB9*	N, C	Novel
4	*CUL1*	N, C **Centrosome**	Korzeniewski et al., 2009
5	*ENC1*	P.M, C.B	Novel
6	*FBXL14*	N, C ,G	Novel
7	*FBXL18*	N, C	Novel
8	*FBXO21*	M	Novel
9	*FBXO31*	**Centrosome**	Vadhvani et al, 2013
10	*FBXO32*	N, C, G	Novel
11	*FBXO4*	N	Novel
12	*FBXO9*	**Centrosome**	
13	*FBXW11*	N, V **Centrosome**	Guderian et al., 2010
14	*FBXW12*	C, P, M	Novel
15	*HECTD2*	N	Novel
16	*HERC3*	C	Novel
17	*KLHL2*	N, V **Centrosome**	Novel
18	*PCGF5*	N	Novel
19	*PELI1*	N, I.F **Spindle pole**	Parketal, 2017
20	*RHOBTB3*	V	Novel
21	*RNF125*	N	Novel
22	*RNF135*	V	Novel
23	*RNF25*	N, C, G	
24	*SPSB1*	N, C, M	Novel
25	*SPSB4*	N, P.M	Novel
26	*TCEB2*	N, C	Novel
27	*TRIM24*	N	Novel
28	*TRIM36*	N, C	Novel
29	*UBE3C*	N, P.M	Novel
30	*VHL*	N , C **Cilium**	Lutz and Burk, 2016
31	*WWP2*	N, C, M **Centrosome**	Akimov et al., 2011

* N; nucleus, C; cytosol, P.M; plasma membrane, C.B; cytoplasmic body, G; Golgi apparatus, M; mitochondria,
V; vesicle, I.F; intermediated filaments

A.


GFP
ANAPC11
ASB3
ASB9
CUL1
ENC1
FBXL14
FBXL18
FBXO21
FBXO31
FBXO32
FBXO4
FBXO9
FBXW11
FBXW12
HECTD2
HERC3
KLHL2
PCGF5
PELI1
RNF125
RNF135
RNF25
SPSB1
SPSB4
TCEB2
TRIM24
TRIM36
UBE3C
VHL
WWP2

B.

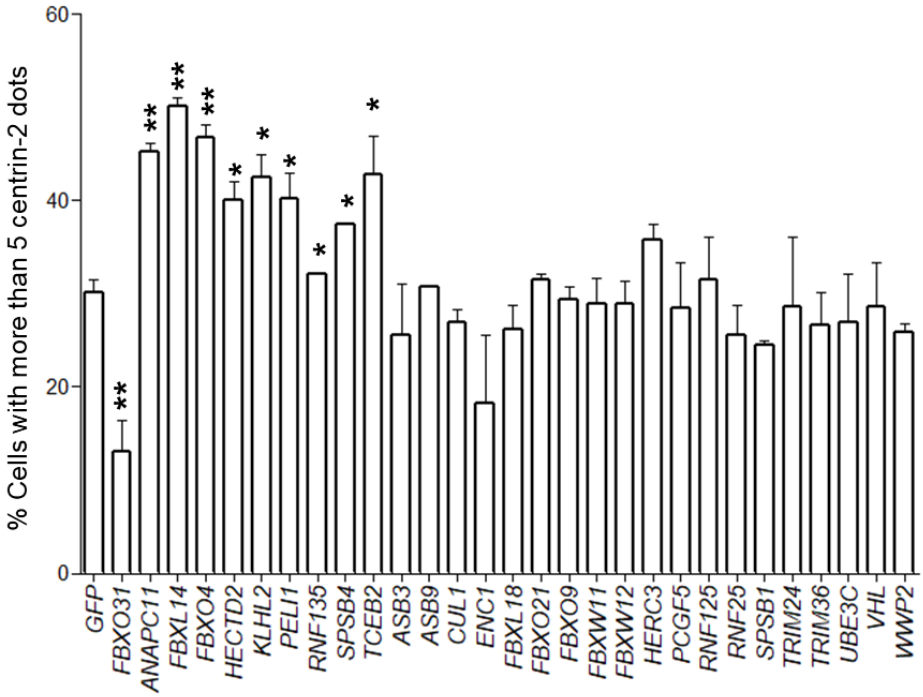


Figure 25. Functional analysis of centrosomal E3 ubiquitin ligases for the suppression of centrosome amplification. U2OS cells were transfected with the indicated expression vectors for 24 hours, and treated with 2 mM of hydroxyurea (HU) for 72 hours. (A) Cells were co-immunostained with antibodies specific to GFP and Centrin-2. Scale bar, 10 μm. (B) The number of cells having 5 more Centrin-2 dot was counted. More than 50 cells per group were counted in 2 independent experiments. Values are presented as the mean ± standard error. *, P< 0.05; **, P< 0.01; ***, P< 0.001.

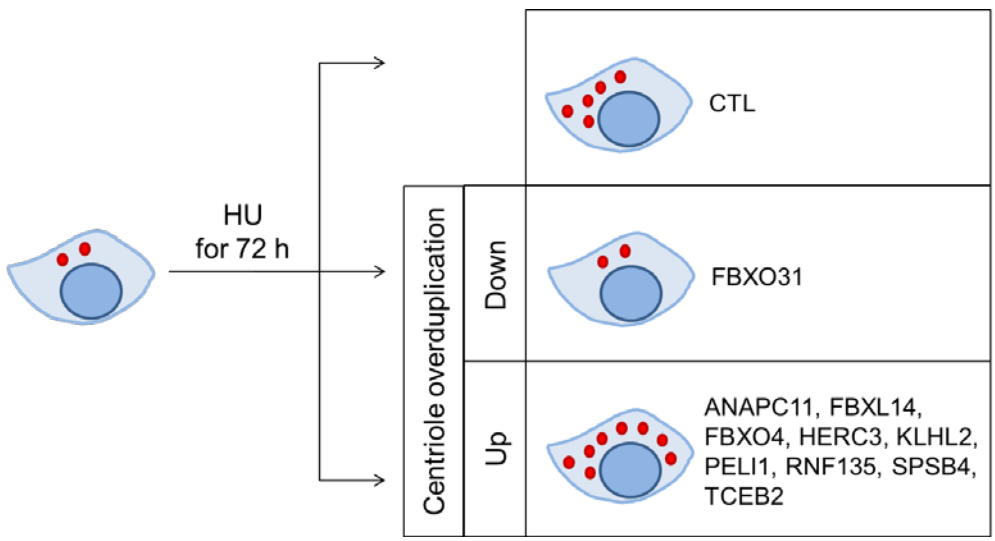


Figure 26. Model of E3 Ub ligase in the regulation of centriole duplication. FBXO31 suppresses centriole overduplication. ANAPC11, FBXL14, FBXO4, HERC3, KLHL2, PELI1, RNF135, SPSB4, and TCEB2 promote centriole amplification, implying that their functions are important for preventing overduplication and regulating the centriole duplication.

Discussion

Centrosome duplication is a strictly regulated process. Centrosome duplication is initiated after centriole disengagement and separation. Once a procentriole grows perpendicularly next to the mother centriole, a new round of centriole assembly is blocked. This called centrosome-intrinsic mechanism (Kleylein-Sohn et al., 2007). Amount of regulatory proteins responsible for centrosome duplication is also critical for proper progression of centrosome duplication (Bettencourt-Dias et al., 2005). PLK4 is a key regulator for centrosome duplication. Overexpression of PLK4 increases centriole number, but depletion of PLK4 induces disrupts in centrosome duplication (Kleylein-Sohn et al., 2007). Thus, PLK4 level should be tightly regulated during the centrosome duplication. The cellular level of PLK4 during centrosome duplication is controlled by several E3 ubiquitin ligases, such as FBXW11/β-TrCP, CUL1, and MIB1, with different mechanisms (Guderian et al., 2010; Korzeniewski et al., 2009; Wang et al., 2016).

So far, extensive studies on centrosome duplication have been performed to find out the function of each centrosomal protein in regulating centrosome duplication. And then researchers identified the target or upstream proteins using IP-combined MASS spectrometry analysis. But, there are limitations since it is difficult to identify centrosomal E3 ubiquitin ligases responsible for centrosome duplication. So, I used different approach to identify the E3 ligases working on the centrosome and analyze the function of the identified E3 ligases in controlling centriole duplication.

Among 226 E3 ubiquitin ligases, 31 E3 ligases, 8 known and 23 novel E3

ligases, were determined to localize to the centrosome by immunostaining. And the functional analysis of the centrosomal E3 ligases to regulate centrosome duplication revealed that FBXO31 suppressed centrosome amplification and ANAPC11, FBXL14, FBXO4, HERC3, KLHL2, PELI1, RNF135, SPSB4, and TCEB2 promoted centrosome amplification, implying that their function are important for preventing overduplication and regulating the centriole duplication.

Among the 31 E3 ligases, only FBXO31 suppressed centrosome amplification. FBXO31 is originally known as a tumor suppressor in breast cancer and binds to the SKP1-CUL1-ROC1 (SCF) complex, which forms a functional SCF-FBXO31 E3 ubiquitin ligase (Johansson et al., 2014). It was reported that CUL1 is required for suppressing centriole overduplication. Depletion or inhibition of CUL1 caused centrosome amplification by affecting PLK4 protein stability (Korzeniewski et al., 2009). Based on these results, it may propose that FBXO31 with CUL1 restrains excessive centriole duplication. However, it remains to be elucidated whether depletion of FBXO31 affects centrosome duplication and FBXO31 with CUL1 is involved in suppressing centriole overduplication by affecting the stability of PLK4. And the substrate of FBXO31 in regulating centrosome duplication has not been revealed. Therefore, the functional and mechanistic study needs to be further analyzed. This is first finding that FBXO31 is implicated in suppressing excessive centriole duplication.

It was previously shown that PELI1 negatively regulates mitotic spindle checkpoint (Park et al., 2017). PELI1 binds to BUBR1 and its overexpression leads to degradation of BUBR1 by K48-mediated polyubiquitination (Park et al., 2017).

BUBR1 is known as a suppressor of centrosome amplification in premature chromatid separation (PCS) syndrome cells that lost the machinery regulating centrosome duplication, resulting in multipolar mitosis and centrosome amplification (Izumi et al., 2009). Here, I observed that overexpression of PELI1 promoted extensive centriole overduplication. Consistent with my result, overexpression of PELI1 increase cell size, induces chromosome aneuploidy *in vivo* and develops tumors (Park et al., 2017), phenomenon of centrosome amplification (Godinho and Pellman, 2014). Taken together, these results may indicate that PELI1 positively regulates centrosome duplication by controlling the stability of BUBR1. However, it remains to be elucidated whether depletion of PEL1 affects centrosome duplication and PELI1 promotes centriole amplification through affecting the stability of BUBR1. Therefore, the functional and mechanistic study needs to be further analyzed.

Several prolonged S phase-arrested E3 ubiquitin ligases did not localize to the centrosome. Among 31, only 8 were found in the centrosome. It may be explained like this. 1) If the E3 ligase is fluctuated during the cell cycle, its centrosomal signals could not be lost by prolonged S phase arrest. 2) If the E3 ligase is a traveling enzyme from cytosol/nuclei to centrosomes, it could not be arrived to the centrosome.

Centrosome amplification is a hallmark of cancer (Godinho and Pellman, 2014). Centrosome abnormalities are observed in many cancer cells such as ovarian, breast prostate, colon, and pancreatic cancer (Hsu et al., 2005; Lingle et al., 1998). Centrosome amplification leads to disrupt in asymmetric division and aneuploidy during mitosis and defects in cell migration, invasion, polarity and signaling in interphase (Godinho and Pellman, 2014). E3 ubiquitin ligases are also closely linked

with cancer (Nakayama and Nakayama, 2006). The E3 ligases are overexpressed or downregulated in cancers. Thus, the functional and mechanistic analysis of centrosomal E3 ligases to control centrosome duplication will provide the information for the mechanism of developing cancer.

Also, it needs to study other centrosomal functions such as centrosome maturation, separation, and ciliogenesis of identified centrosomal E3 ligases.

Conclusion

This study has been focused on identifying PTM enzymes that function in cilia formation and centrosome duplication through deacetylation and ubiquitination, respectively. In proliferating cells, centrosome duplicates throughout the cell cycle, but in quiescent cells, the centrosome serves as a basal body for cilia formation. Therefore, these centrosomal proteins are dynamically subject to various protein modifications.

In the chapter 1, I investigated the novel functions of HDAC in the assembly and elongation of cilium. HDAC3 and HDAC8 are required for the cilium formation and HDAC8 is essential for the cilium elongation. This is the first study to report that deacetylases are required for the assembly and elongation of the cilium.

In the chapter 2, I examined how E3 ubiquitin ligase is involved in centrosome duplication. First, I identified 31 E3 ubiquitin ligases located at the centrosome among 226 E3 Ub ligases. Second, of the 31 identified E3 Ub ligases, I found that 10 E3 Ub ligases are candidates for the regulation of centrosome duplication, FBXO31 as the suppressor of centriole overduplication and ANAPC11, FBXL14, FBXO4, HERC3, KLHL2, PELI1, RNF135, SPSB4, and TCEB2 as the promoters of centriole overduplication. So, the further functional studies of 10 E3 ligases will provide interesting clues for understanding genomic instability, and further cancer development.

References

Akella, J.S., Wloga, D., Kim, J., Starostina, N.G., Lyons-Abbott, S., Morrissette, N.S., Dougan, S.T., Kipreos, E.T., and Gaertig, J. (2010). MEC-17 is an alpha-tubulin acetyltransferase. Nature *467*, 218-222.

Asante, D., Maccarthy-Morrogh, L., Townley, A.K., Weiss, M.A., Katayama, K., Palmer, K.J., Suzuki, H., Westlake, C.J., and Stephens, D.J. (2013). A role for the Golgi matrix protein giantin in ciliogenesis through control of the localization of dynein-2. Journal of cell science *126*, 5189-5197.

Bedford, L., Lowe, J., Dick, L.R., Mayer, R.J., and Brownell, J.E. (2011). Ubiquitin-like protein conjugation and the ubiquitin-proteasome system as drug targets. Nat Rev Drug Discov *10*, 29-46.

Bettencourt-Dias, M., and Glover, D.M. (2007). Centrosome biogenesis and function: centrosomics brings new understanding. Nat Rev Mol Cell Biol *8*, 451-463.

Bettencourt-Dias, M., Hildebrandt, F., Pellman, D., Woods, G., and Godinho, S.A. (2011). Centrosomes and cilia in human disease. Trends Genet *27*, 307-315.

Bettencourt-Dias, M., Rodrigues-Martins, A., Carpenter, L., Riparbelli, M., Lehmann, L., Gatt, M.K., Carmo, N., Balloux, F., Callaini, G., and Glover, D.M. (2005).

SAK/PLK4 is required for centriole duplication and flagella development. Curr Biol *15*, 2199-2207.

Cajanek, L., Glatter, T., and Nigg, E.A. (2015). The E3 ubiquitin ligase Mib1 regulates Plk4 and centriole biogenesis. Journal of cell science *128*, 1674-1682.

Cohen, H.Y., Lavu, S., Bitterman, K.J., Hekking, B., Imahiyerobo, T.A., Miller, C., Frye, R., Ploegh, H., Kessler, B.M., and Sinclair, D.A. (2004). Acetylation of the C terminus of Ku70 by CBP and PCAF controls Bax-mediated apoptosis. Mol Cell *13*, 627-638.

Deshaies, R.J., and Joazeiro, C.A. (2009). RING domain E3 ubiquitin ligases. Annu Rev Biochem *78*, 399-434.

Fliegauf, M., Benzing, T., and Omran, H. (2007). When cilia go bad: cilia defects and ciliopathies. Nat Rev Mol Cell Biol *8*, 880-893.

Frescas, D., and Pagano, M. (2008). Deregulated proteolysis by the F-box proteins SKP2 and beta-TrCP: tipping the scales of cancer. Nat Rev Cancer *8*, 438-449.

Fukasawa, K. (2007). Oncogenes and tumour suppressors take on centrosomes. Nat Rev Cancer *7*, 911-924.

Glickman, M.H., and Ciechanover, A. (2002). The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. Physiol Rev *82*, 373-428.

Godinho, S.A., and Pellman, D. (2014). Causes and consequences of centrosome abnormalities in cancer. Philos Trans R Soc Lond B Biol Sci *369*.

Goetz, S.C., and Anderson, K.V. (2010). The primary cilium: a signalling centre during vertebrate development. Nat Rev Genet *11*, 331-344.

Goetz, S.C., Liem, K.F., Jr., and Anderson, K.V. (2012). The spinocerebellar ataxia-associated gene Tau tubulin kinase 2 controls the initiation of ciliogenesis. Cell *151*, 847-858.

Guderian, G., Westendorf, J., Uldschmid, A., and Nigg, E.A. (2010). Plk4 trans-autophosphorylation regulates centriole number by controlling betaTrCP-mediated degradation. Journal of cell science *123*, 2163-2169.

Haberland, M., Montgomery, R.L., and Olson, E.N. (2009). The many roles of histone deacetylases in development and physiology: implications for disease and therapy. Nat Rev Genet *10*, 32-42.

Hsu, L.C., Kapali, M., DeLoia, J.A., and Gallion, H.H. (2005). Centrosome abnormalities in ovarian cancer. Int J Cancer *113*, 746-751.

Ishikawa, H., and Marshall, W.F. (2011). Ciliogenesis: building the cell's antenna. Nat Rev Mol Cell Biol *12*, 222-234.

Ishikawa, H., and Marshall, W.F. (2017). Intraflagellar Transport and Ciliary Dynamics. Cold Spring Harb Perspect Biol *9*.

Izumi, H., Matsumoto, Y., Ikeuchi, T., Saya, H., Kajii, T., and Matsuura, S. (2009). BubR1 localizes to centrosomes and suppresses centrosome amplification via regulating Plk1 activity in interphase cells. Oncogene *28*, 2806-2820.

Jensen, E.D., Schroeder, T.M., Bailey, J., Gopalakrishnan, R., and Westendorf, J.J. (2008). Histone deacetylase 7 associates with Runx2 and represses its activity during osteoblast maturation in a deacetylation-independent manner. J Bone Miner Res *23*, 361-372.

Jin, J., Cardozo, T., Lovering, R.C., Elledge, S.J., Pagano, M., and Harper, J.W. (2004). Systematic analysis and nomenclature of mammalian F-box proteins. Genes Dev *18*, 2573-2580.

Johansson, P., Jeffery, J., Al-Ejeh, F., Schulz, R.B., Callen, D.F., Kumar, R., and Khanna, K.K. (2014). SCF-FBXO31 E3 ligase targets DNA replication factor Cdt1 for proteolysis in the G2 phase of cell cycle to prevent re-replication. J Biol Chem *289*, 18514-18525.

Keeling, J., Tsiokas, L., and Maskey, D. (2016). Cellular Mechanisms of Ciliary Length Control. Cells *5*.

Kim, J., Lee, K., and Rhee, K. (2015a). PLK1 regulation of PCNT cleavage ensures fidelity of centriole separation during mitotic exit. Nat Commun *6*, 10076.

Kim, S., Lee, K., Choi, J.H., Ringstad, N., and Dynlacht, B.D. (2015b). Nek2 activation of Kif24 ensures cilium disassembly during the cell cycle. Nat Commun *6*, 8087.

Kim, T.S., Park, J.E., Shukla, A., Choi, S., Murugan, R.N., Lee, J.H., Ahn, M., Rhee, K., Bang, J.K., Kim, B.Y., *et al.* (2013). Hierarchical recruitment of Plk4 and regulation of centriole biogenesis by two centrosomal scaffolds, Cep192 and Cep152. Proc Natl Acad Sci U S A *110*, E4849-4857.

Kleylein-Sohn, J., Westendorf, J., Le Clech, M., Habedanck, R., Stierhof, Y.D., and Nigg, E.A. (2007). Plk4-induced centriole biogenesis in human cells. Dev Cell *13*, 190-202.

Kobayashi, T., Nakazono, K., Tokuda, M., Mashima, Y., Dynlacht, B.D., and Itoh, H. (2017). HDAC2 promotes loss of primary cilia in pancreatic ductal adenocarcinoma. EMBO Rep *18*, 334-343.

Korzeniewski, N., Zheng, L., Cuevas, R., Parry, J., Chatterjee, P., Anderton, B., Duensing, A., Munger, K., and Duensing, S. (2009). Cullin 1 functions as a centrosomal suppressor of centriole multiplication by regulating polo-like kinase 4 protein levels. Cancer Res *69*, 6668-6675.

Kuhns, S., Schmidt, K.N., Reymann, J., Gilbert, D.F., Neuner, A., Hub, B., Carvalho, R., Wiedemann, P., Zentgraf, H., Erfle, H., *et al.* (2013). The microtubule affinity regulating kinase MARK4 promotes axoneme extension during early ciliogenesis. J Cell Biol *200*, 505-522.

Li, L., and Yang, X.J. (2015). Tubulin acetylation: responsible enzymes, biological functions and human diseases. Cell Mol Life Sci *72*, 4237-4255.

Li, W., Bengtson, M.H., Ulbrich, A., Matsuda, A., Reddy, V.A., Orth, A., Chanda, S.K., Batalov, S., and Joazeiro, C.A. (2008). Genome-wide and functional annotation of human E3 ubiquitin ligases identifies MULAN, a mitochondrial E3 that regulates the organelle's dynamics and signaling. PloS one *3*, e1487.

Li, Y., and Hu, J. (2011). Small GTPases and cilia. Protein Cell *2*, 13-25.

Ling, H., Peng, L., Seto, E., and Fukasawa, K. (2012). Suppression of centrosome duplication and amplification by deacetylases. Cell Cycle *11*, 3779-3791.

Lingle, W.L., Lutz, W.H., Ingle, J.N., Maihle, N.J., and Salisbury, J.L. (1998). Centrosome hypertrophy in human breast tumors: implications for genomic stability and cell polarity. Proc Natl Acad Sci U S A *95*, 2950-2955.

Loukil, A., Tormanen, K., and Sutterlin, C. (2017). The daughter centriole controls ciliogenesis by regulating Neurl-4 localization at the centrosome. J Cell Biol *216*, 1287-1300.

Michaud, E.J., and Yoder, B.K. (2006). The primary cilium in cell signaling and cancer. Cancer Res *66*, 6463-6467.

Miyamoto, T., Hosoba, K., Ochiai, H., Royba, E., Izumi, H., Sakuma, T., Yamamoto, T., Dynlacht, B.D., and Matsuura, S. (2015). The Microtubule-Depolymerizing Activity of a Mitotic Kinesin Protein KIF2A Drives Primary Cilia Disassembly Coupled with Cell Proliferation. Cell Rep.

Moser, J.J., Fritzler, M.J., and Rattner, J.B. (2009). Primary ciliogenesis defects are associated with human astrocytoma/glioblastoma cells. BMC Cancer *9*, 448.

Nachury, M.V., Loktev, A.V., Zhang, Q., Westlake, C.J., Peranen, J., Merdes, A., Slusarski, D.C., Scheller, R.H., Bazan, J.F., Sheffield, V.C., *et al.* (2007). A core complex of BBS proteins cooperates with the GTPase Rab8 to promote ciliary membrane biogenesis. Cell *129*, 1201-1213.

Nakayama, K.I., and Nakayama, K. (2006). Ubiquitin ligases: cell-cycle control and cancer. Nat Rev Cancer *6*, 369-381.

Nigg, E.A., and Raff, J.W. (2009). Centrioles, centrosomes, and cilia in health and disease. Cell *139*, 663-678.

Ohta, M., Ashikawa, T., Nozaki, Y., Kozuka-Hata, H., Goto, H., Inagaki, M., Oyama, M., and Kitagawa, D. (2014). Direct interaction of Plk4 with STIL ensures formation of a single procentriole per parental centriole. Nat Commun *5*, 5267.

Pan, J., and Snell, W.J. (2014). Organelle size: a cilium length signal regulates IFT cargo loading. Curr Biol *24*, R75-78.

Park, J., Park, H.Y., Kim, S., Kim, H.S., Park, J.Y., Go, H., and Lee, C.W. (2017). Pellino 1 inactivates mitotic spindle checkpoint by targeting BubR1 for ubiquitinational degradation. Oncotarget *8*, 32055-32067.

Peters, J.M. (2006). The anaphase promoting complex/cyclosome: a machine designed to destroy. Nat Rev Mol Cell Biol *7*, 644-656.

Petroski, M.D., and Deshaies, R.J. (2005). Function and regulation of cullin-RING ubiquitin ligases. Nat Rev Mol Cell Biol *6*, 9-20.

Plotnikova, O.V., Nikonova, A.S., Loskutov, Y.V., Kozyulina, P.Y., Pugacheva, E.N., and Golemis, E.A. (2012). Calmodulin activation of Aurora-A kinase (AURKA) is required during ciliary disassembly and in mitosis. Mol Biol Cell *23*, 2658-2670.

Pugacheva, E.N., Jablonski, S.A., Hartman, T.R., Henske, E.P., and Golemis, E.A. (2007). HEF1-dependent Aurora A activation induces disassembly of the primary cilium. Cell *129*, 1351-1363.

Puklowski, A., Homsi, Y., Keller, D., May, M., Chauhan, S., Kossatz, U., Grunwald, V., Kubicka, S., Pich, A., Manns, M.P., *et al.* (2011). The SCF-FBXW5 E3-ubiquitin ligase is regulated by PLK4 and targets HsSAS-6 to control centrosome duplication. Nat Cell Biol *13*, 1004-1009.

Ran, J., Yang, Y., Li, D., Liu, M., and Zhou, J. (2015). Deacetylation of alpha-tubulin and cortactin is required for HDAC6 to trigger ciliary disassembly. Sci Rep *5*, 12917.

Sanchez, I., and Dynlacht, B.D. (2016). Cilium assembly and disassembly. Nat Cell Biol *18*, 711-717.

Schmidt, K.N., Kuhns, S., Neuner, A., Hub, B., Zentgraf, H., and Pereira, G. (2012). Cep164 mediates vesicular docking to the mother centriole during early steps of ciliogenesis. J Cell Biol *199*, 1083-1101.

Seeley, E.S., Carriere, C., Goetze, T., Longnecker, D.S., and Korc, M. (2009). Pancreatic cancer and precursor pancreatic intraepithelial neoplasia lesions are devoid of primary cilia. Cancer Res *69*, 422-430.

Seo, M.Y., Jang, W., and Rhee, K. (2015). Integrity of the Pericentriolar Material Is Essential for Maintaining Centriole Association during M Phase. PloS one *10*, e0138905.

Serrador, J.M., Cabrero, J.R., Sancho, D., Mittelbrunn, M., Urzainqui, A., and Sanchez-Madrid, F. (2004). HDAC6 deacetylase activity links the tubulin cytoskeleton with immune synapse organization. Immunity *20*, 417-428.

Shida, T., Cueva, J.G., Xu, Z., Goodman, M.B., and Nachury, M.V. (2010). The major alpha-tubulin K40 acetyltransferase alphaTAT1 promotes rapid ciliogenesis and efficient mechanosensation. Proc Natl Acad Sci U S A *107*, 21517-21522.

Sung, C.H., and Leroux, M.R. (2013). The roles of evolutionarily conserved functional modules in cilia-related trafficking. Nat Cell Biol *15*, 1387-1397.

Swatek, K.N., and Komander, D. (2016). Ubiquitin modifications. Cell Res *26*, 399-422.

Wang, L., Lee, K., Malonis, R., Sanchez, I., and Dynlacht, B.D. (2016). Tethering of an E3 ligase by PCM1 regulates the abundance of centrosomal KIAA0586/Talpid3

and promotes ciliogenesis. Elife *5*.

Welcker, M., and Clurman, B.E. (2008). FBW7 ubiquitin ligase: a tumour suppressor at the crossroads of cell division, growth and differentiation. Nat Rev Cancer *8*, 83-93.

Yamauchi, Y., Boukari, H., Banerjee, I., Sbalzarini, I.F., Horvath, P., and Helenius, A. (2011). Histone deacetylase 8 is required for centrosome cohesion and influenza A virus entry. PLoS Pathog *7*, e1002316.

Zheng, N., Schulman, B.A., Song, L., Miller, J.J., Jeffrey, P.D., Wang, P., Chu, C., Koepp, D.M., Elledge, S.J., Pagano, M., *et al.* (2002). Structure of the Cul1-Rbx1-Skp1-F boxSkp2 SCF ubiquitin ligase complex. Nature *416*, 703-709.

Zheng, N., Wang, Z., and Wei, W. (2016). Ubiquitination-mediated degradation of cell cycle-related proteins by F-box proteins. Int J Biochem Cell Biol *73*, 99-110.

Zhou, X., Fan, L.X., Li, K., Ramchandran, R., Calvet, J.P., and Li, X. (2014). SIRT2 regulates ciliogenesis and contributes to abnormal centrosome amplification caused by loss of polycystin-1. Hum Mol Genet *23*, 1644-1655.

국문초록

중심체는 두 개의 중심립과 그를 둘러싼 중심구 단백질로 구성되어 있다. 세포주기 동안, 중심체는 다양한 역할을 수행한다. 간기 때는 미세소관 조직화 중심으로써의 역할을 하고, 분열기 동안에는 방추사를 형성하여 복제된 염색체를 두 개의 딸세포로 동일하게 나눠준다. 세포 휴지기 때에는 기저체로 변환되어 섬모를 형성하는 역할을 수행한다. 이러한 기능들을 수행하기 위해서, 중심체 단백질들은 세포주기 동안 인산화, 유비퀴틴화, 아세틸화 등 다양한 변화를 겪는다. 그 변화들은 중심체 단백질의 활성과 안정성을 조절하여 중심체주기에 영향을 미친다. 이 연구에서는 섬모주기에서 히스톤 탈아세틸화 효소의 기능과 중심체 복제에서 E3 유비퀴틴 중합효소의 기능을 살펴보았다.

제 1장에서는 섬모의 형성과 신장과정 동안 class 1 히스톤 탈아세틸화 효소의 기능에 대해 연구하였다. 히스톤 탈아세틸화 효소는 본래 히스톤을 탈아세틸화 시키면서 유전자의 발현을 조절하는 것으로 알려졌다. 히스톤 탈아세틸화 효소는 또한, 히스톤 단백질이 아닌 단백질을 타겟하고 탈아세틸화 시켜서 타겟 단백질의 활성과 기능에 영향을 주는 것으로도 알려졌다. 기존 연구에서 어떤 히스톤 탈아세틸화 효소들은 중심체에 존재하고 중심체와 섬모주기에서 기능을 한다는 것이 밝혀졌다. 그러나 지금까지 섬모의 형성에 관여하는 탈아세틸화 효소는 알려진바 없다. 따라서 본 연구에서 섬모형성과 신장에 관여하는 class 1 히스톤 탈아세틸화 효소의 기능을 연구하였다. 그 결과, HDAC3와 HDAC8이 섬모형성에 관여하고, HDAC8이 섬모의 신장에 관여한다는 것을 관찰하였고, 이것은 히스톤 탈

아세틸화 효소가 섬모의 형성과 신장 과정에 역할을 한다는 것을 밝힌 첫 번째 연구이다.

제 2장에서는 중심체 복제에 관여하는 중심체 E3 유비퀴틴 중합효소의 기능을 연구하였다. 중심체 복제에 관여하는 중심체 단백질들은 E3 유비퀴틴 중합효소에 의해 급격한 분해를 겪는다. 그들은 중심체 단백질들을 유비퀴틴화 시키고, 중심체 단백질들의 활성과 안정성을 조절하여, 중심체 복제 과정에 영향을 준다. 본 연구에서는 226개의 E3 유비퀴틴 중합효소 중에서 중심체에 위치하는 E3 유비퀴틴 중합효소를 선별하고, 중심체 복제 과정에 영향을 주는 E3 유비퀴틴 중합효소의 기능에 대해 살펴보았다. 그들 중에서, 7개의 알려진 E3 중합효소와 24개의 새로운 E3 중합효소가 중심체에 위치함을 관찰하였다. 총 31개의 E3 중합효소 중에서 FBXO31이 중심체 과복제를 억제하는데 관여하며, ANAPC11, FBXL14, FBXO4, HERC3, KLHL2, PELI1, RNF135, SPSB4, TCEB2가 중심체 과복제를 촉진한다는 것을 관찰하였다. 중심체에 존재하는 E3 중합효소를 포괄적으로 관찰하고, 그의 기능을 살펴봄으로써 중심체 E3 중합효소의 기능에 대해 좀더 포괄적으로 연구할 수 있도록 도와주는 연구라고 생각된다.

주요어: 중심체, 섬모, 중심체 복제, 단백질번역 후 변형, 히스톤 탈아세틸화 효소, E3 유비퀴틴 중합효소, 세포주기

학 번: 2012-30885